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Acta Pathologica et Microbiologica Scandinavica is intended for the prompt publication of original research in the fields of pathology microbiology and immunology. It is included in *Current Contents*, *Excerpta Medica*, and *Medlars*.

Acta Pathologica et Microbiologica Scandinavica is a nonprofit making scientific journal. Since 1924 it has been published by the Scandinavian Societies for Medical Microbiology and Pathology. It appears in three sections. Section A. Pathology Section B. Microbiology and Section C. Immunology.

Acta Pathologica et Microbiologica Scandinavica has subscribers in more than seventy countries throughout the world with a wide readership in the major research institutes, hospitals, laboratories, and specialist libraries.

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At present one annual volume of Section A, one of Section B and one of Section C (each section consisting of 6 issues appearing bimonthly) will contain a total of approximately 1400 pages. During the past few years approximately five free supplements have been issued annually. These supplements will be delivered separately to the subscribers by surface mail at no extra charge. The subscription price is:

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Second class postage paid at Jamaica, N.Y., Airfreight and mailing in the U.S. by Publications Expediting Inc. Elmont, NY 11003. Printed in Denmark.

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FOCAL SPONTANEOUS ALTERATIONS AND LOSS OF ENDOTHELIAL CELLS IN RABBIT AORTA

EDUAR SVENDSEN and LEIV JØRGENSEN

Institute of Medical Biology Department of Morphology University of Tromsø, Norway

Svensen, E. & Jørgensen L. Focal spontaneous alterations and loss of endothelial cells in rabbit aorta. Acta path. microbiol. scand. Sect. A, 86 1-13 1978.

Rabbit aortas were examined by light, scanning, and transmission electron microscopy to observe the morphological steps in focal endothelial cell desquamation. The aortas were not subjected to any instrumentation prior to fixation by either immersion alone or combined perfusion and immersion. In areas where boundary layer separation of flow with eddy formation is known to occur many elongated protruding cells were observed, some obviously partly loosened from the basement membrane. Some of the latter cells appeared to be attached by re-ends and so be twisted longitudinally. Single cells, or even sheets of cells, were completely detached. The breaks seemed to have taken place close to and parallel with intercellular junctions, but not within these structures. Platelets had reacted both with injured cells and the denuded internal surface. Thus, the sequence appears to be protrusion of cell body, partial loosening of cells from the basement membrane, breaks near the intercellular junctions, twisting of cells, and complete detachment.

Key words: Aorta, endothelial cells, focal alterations, rabbit.

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Received 6/77 Accepted 31 vii 77

Boutier *et al.* (3) found endothelial cells in venous blood samples from man, and Hladovc & Rossmann (8) were able to isolate endothelial cells from rat blood taken from the right side of the heart. Some of these cells had obviously lost their nuclei. Boutier *et al.* (3) found a higher number of circulating endothelial cells in patients with manifest or potential thrombotic disease and considered the presence of these cells to be an indication of vascular injury.

It is uncertain whether these cells are derived from the arterial or venous side of the vascular bed or from both.

As to the arterial vascular bed, several authors (20, 21, 23, 29, 30) describe various intracellular endothelial alterations and sub-endothelial blebs or edema between the periph-eral cell membrane and the basement membrane. In focal areas the endothelial cells are lost (10, 11, 26). The endothelial changes and the focal loss may be accompanied by platelet accumulation (1, 10, 11). Tødder & Shorey observed subendothelial blebs also of venous endothelium in rabbits (28).

By scanning electron microscopy Shimamoto *et al.* (19) observed protruding longitudinal structures on the arterial surface and

called them "bridge like structures" *Christensen & Garbarsch* (4) considered the "bridge-like structures" to be shrinkage artefacts. *Sunaga et al* (24) and *Nelson et al* (14) felt that these structures were distorted endothelial cells. *Elmér et al* (5) found similar structures in rat aortas following ischemia and suggested that the "bridges" may have been processes of endothelial cells developing as a response to cell injury. *Reidy & Bowyer* (16) depicted similar structures at the branching sites of rabbit aortas and considered the structures to be injured cells. The cause of the injury was thought to be haemodynamic forces on the arterial wall. The same authors demonstrated similar structures in rabbit aortas after α administration of endotoxin (17).

The aim of the present paper is to study how endothelial cells are detached from the aortas of rabbits. What are the changes which precede the detachment of endothelial cells? Do the so-called "bridge like structures" reflect injured endothelial cells which may be come loosened?

MATERIALS AND METHODS

Altogether 24 rabbits (albino and Dutch strain) were used. All animals were raised on a lipid poor standard diet of commercial food pellets for rabbits (Karin Pellets, Felleskjøpet Trondheim Norway). Water was given *ad libitum*.

Nine animals got a diet consisting of crushed

pellets mixed up with pure cholesterol-powder (BHD Chemicals Ltd. Poole England) to a concentration of 2 per cent by weight for 1 or 2 days. (Table 1)

The animals ate from 80 up to 150 g of the diet per day i.e. 16 to 30 g pure cholesterol. The animals were sacrificed without further experimentation and aorta specimens were either fixed by immersion (14 rabbits) or by perfusion and immersion combined (10 rabbits) (Table 1)

The animals on which the aorta specimens were to be fixed by immersion alone were killed by an intravenous overdose of Nembutal "Abbot". Thorax and abdomen were rapidly opened and the aorta carefully removed and opened along the ventral aspect with a pair of scissors with a blunt point.

For light, scanning, and transmission electron microscopy the aorta was rinsed in buffered Tyrodes solution and specimens were taken from standardized sites (25) (Fig 1)

In rabbits in which the aorta was to be perfused the thorax was rapidly opened under intravenous anesthesia with Nembutal "Abbot". A flexible polyethylen cannula was placed in the ascending part of the aorta through an incision made in the left ventricle. A ligature was placed at the beginning of the aorta. Another incision was made in the right ventricle.

The blood was washed out with phosphate buffered Tyrodes solution with pH 7.4 and osmolarity of approximately 300 mOsmol/kg. The perfusion was continued with phosphate buffered glutaraldehyde 2 per cent, pH 7.4 for 10 minutes the pressure was kept at 80 mm Hg during the whole procedure. The aorta was then carefully removed, opened along the ventral aspect, and specimens were taken for light, scanning and transmission electron microscopy from standardized sites (Fig 1)

Specimens for light microscopy only were cut

TABLE 1 Grouping of Animals and Fixation Procedures. The Table gives the Number of Animals Subjected to Immersion and Perfusion Fixation Procedures in the Three Treatment Groups

Feeding	Type of fixation of aorta		
	Immersion alone No. of animals	Perfusion and immersion No. of animals	Total No of animals
No cholesterol added to the diet	6	3	9
2 per cent cholesterol added to the diet for 1 day prior to kill	4	4	8
2 per cent cholesterol added to the diet for 2 days prior to kill	4	3	7
Total	14	10	24

bedded in Histowax, granulated, (Histo-lab Bethlehens Trausing Ltd., Gothenburg, Sweden) and 4 μ thick sections were cut edgewise and stained with haematoxylin-eosin and Victoria blue. Specimens for scanning electron microscopy whether the aorta had been perfused or not, were fixed in phosphate buffered 2 per cent glutaraldehyde for 2 hours at room temperature, dehydrated in increasing concentrations of ethanol and dried in a Hitachi critical point dryer using CO_2 . The specimens were glued on to metal stubs with conducting paint (Ladd Research Industries, Inc., Burlington, Vermont, USA) and covered with a thin layer of gold, using a scanning electron microscopy sputtering coating unit (E 5000 Polaron Equipment, Ltd., Watford, Hertfordshire, England). The specimens were examined in a Hitachi HH3-2R scanning electron microscope.

Specimens for transmission electron microscopy whether the aorta had been perfused or not, were fixed in phosphate buffered 2 per cent glutaraldehyde with pH 7.4 for 2 hours at room temperature and post-fixed in 1 per cent osmium tetroxide for 1½ hours, dehydrated in increasing concentrations of ethanol and embedded in epon. The specimens were screened by semithin sections cut edgewise and stained with Toluidin blue. Ultrathin sections were cut on a Reichert type 700141 microtome. The sections were placed on metal grids and examined in a Hitachi HU 12 transmission electron microscope.

RESULTS

Light microscopy of both Histowax-embedded and epon-embedded transverse sections showed in area 3 (Fig. 1) a regular pattern of intimal folds with the endothelial surface intact. In the other sampled areas (Fig. 1) pits were seen as earlier described (25). Particularly in areas of pits the nucleus of several endothelial cells were protruding or bulging into the lumen (Fig. 2). Some of the endothelial cells were partly detached, apparently loosened from the underlying intima (Fig. 3) or projected into the lumen, the cell being attached at only one point at the cell periphery. Areas of intima devoid of endothelial cells were occasionally encountered.

Scanning electron microscopy confirmed the findings from light microscopy. Regular intimal folds (Fig. 4) and pits (Fig. 5) were found in perfused as well as in non-perfused animals, although the folds appeared less pronounced in the former.

In areas of regular folds the endothelial

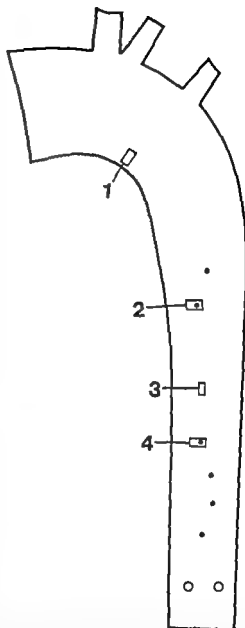
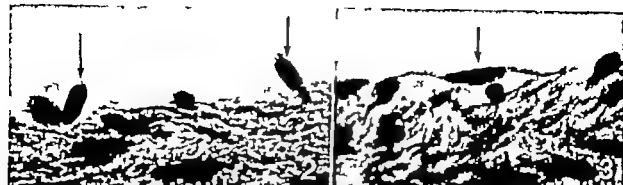


Fig. 1 Diagrammatic illustration of the rabbit aorta. The framed areas indicate the sites of sampling for light, scanning and transmission electron microscopy.

surface was smooth (Fig. 4). In areas of pits, the endothelial layer was altered, particularly in the lower curvature of the aortic arch. Protruding elongated cells were commonly found (Fig. 5). It appeared as if the central



body of the cells with the nucleus formed a crest bulging into the lumen, or as if the central parts of the cell were loosened leaving the cell attached by two "ends" (Fig. 6). The impression of cells being attached only by their "ends" was strengthened by the observation of spiral-like configurations of the peripheral parts of the cell body as if the cells had been twisted around their longitudinal axes (Figs. 7 and 8). In some of the apparently twisted cells the cell membrane seemed ruptured, the nucleus protruding through the rupture (Fig. 9) and in some cases the nucleus seemed to have been lost (Fig. 10).

Infrequently we found one of the elongated ends of the endothelial cells ruptured. The cell projected into the lumen as if it had been pendulating in the flow. Close by the cell had left a sharply outlined oval bed between intact endothelial cells (Fig. 11). More frequently we found similar oval defects without any cells attached, probably representing "beds" left by completely detached single endothelial cells (Fig. 12). The edges of these beds often had double contours (Fig. 13).

Single platelets or groups of platelets were found both in association with protruding or projecting endothelial cells, in the bottom or at the edges of some of the beds (Figs. 10, 14 and 15). Particularly in the lesser curvature

of the aortic arch completely denuded areas were seen, sometimes partly covered by a platelet thrombus (Fig. 16).

Transmission electron microscopy also showed mostly unaltered endothelial cells in area 3 (Fig. 1). In specimens from the other sampled areas altered endothelial cells were often encountered. Severe signs of injury included villi-like processes and ruptured plasma membranes (Figs. 17 and 18). The cytoplasm showed either increased (Fig. 17) or decreased electron density (Fig. 18). The mitochondria were swollen and the cells often vacuolated (Figs. 17 and 18). The perinuclear space was widened and sometimes ruptures of the perinuclear membranes could be seen. The cell nucleus was generally of irregular shape and its chromatin could either be more or less electron dense than normally. The nuclei of these injured cells often protruded into the lumen (Fig. 17).

The first signs of cell desquamation as observed by transmission electron microscopy were subendothelial blebs (Figs. 17 and 19) most often present below the nuclear regions

Fig. 2 From the lesser curvature of the aortic arch. The nuclei of two endothelial cells are "bulging" into the lumen (arrow). Histowax-embedded. Haematoxylin-eosin. 3500 \times .

Fig. 3 From the lesser curvature of the aortic arch. An endothelial cell (arrow) is loosened from the underlayer but apparently still attached at the "ends". Histowax-embedded. Haematoxylin-eosin. 1500 \times .

Fig. 4 Area in rabbit aorta between neighbouring orifices of the intercostal arteries on the same side. Regular intimal folds are seen. Cell junctions are visible (arrow). Perfused specimen. 400 \times .

Fig. 5 From the lesser curvature of the aortic arch. A pit (P) and curved folds (F) are seen. Note the abundance of protruding cells "bridging" across gulches between folds (arrow). Perfused specimen. 420 \times .

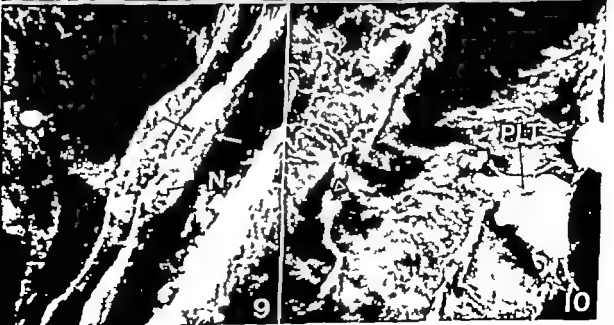
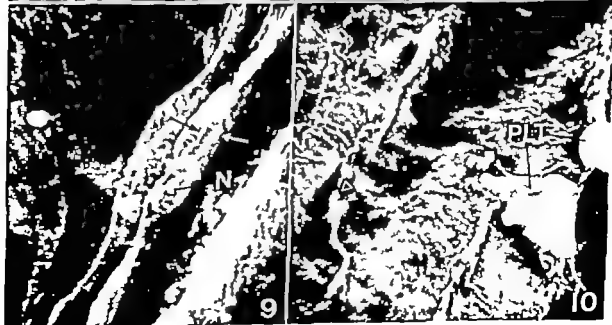
Fig. 6 From the lesser curvature of the aortic arch. An endothelial cell (E) is protruding into the lumen. The central parts of the cell are loosened (arrow) leaving the cell attached only by the two ends. 11300 \times .

Fig. 7 From the lesser curvature of the aortic arch. The peripheral parts of the endothelial cells (E) appear to be twisted (arrows). 3730 \times .

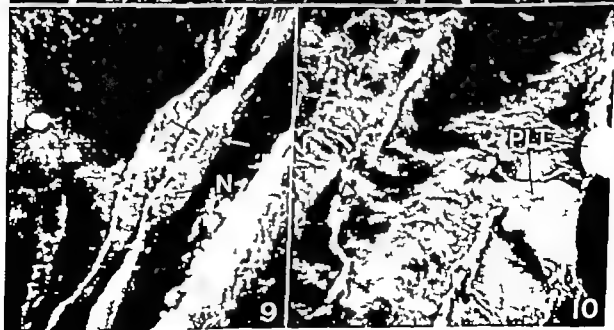
Fig. 8 From the lesser curvature of the aortic arch. Detail from twisted "part" of an endothelial cell. 11700 \times .

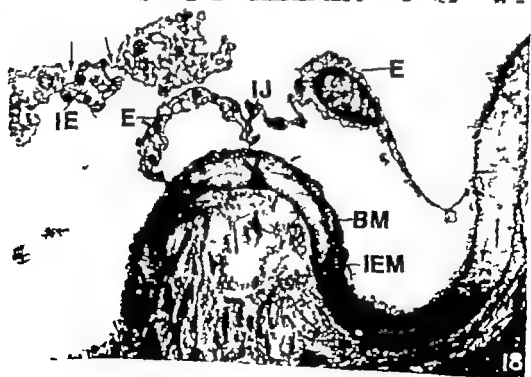
Fig. 9 From the lesser curvature of the aortic arch. Two endothelial cells are loosened from the underlayer. The cell to the right shows a rupture of the cell membrane (arrow) and the nucleus (N) with indentations is visible as hernia on the cell surface. 3900 \times .

Fig. 10 From the lesser curvature of the aortic arch. An endothelial cell nucleus has fallen out and only depression is left in the cytoplasm (triangle). The peripheral parts of the cell body appear to be twisted. The lower of the two platelets (PLT) has adhered to and spread out on the injured endothelium. The upper platelet has kept its rounded form. 3700 \times .









of the cells. At such cells the basement membrane was separated from the outer plasma membrane and often the cell appeared bulging although it was still attached to neighbouring cells at intercellular junctions (Figs. 18 and 19). In some of these cells the peripheral parts of the cytoplasm showed waist like constrictions (Fig. 20) a feature probably corresponding to the spiral like configuration observed by scanning electron microscopy.

Some endothelial cells seemed to have been detached almost completely from the basement membrane, and were joined only by one end to a neighbouring cell. Other cells presented ruptures of the cell bodies near to, but not within, the intercellular junctions (Fig. 21). In such cases small parts of the cell in association with the cell junction were left in situ (Fig. 21). At the edge of denuded areas, similar parts of cytoplasm from ruptured endothelial cells occurred at the intercellular

junctions, which generally seemed to remain intact (Fig. 22).

A quantitative morphometric study of the described alterations is in preparation. Preliminary results obtained from our scanning electron microscopic pictures have so far revealed that protruding cells are more frequently present in sampling areas 1, 2 and 4 than in sampling area 3 (Fig. 1). All the changes described could be detected in most of the animals, whether they had been on a cholesterol-containing diet or not.

No qualitative difference was noted between changes in aortas that had been perfused and those that were fixed by immersion. However the perfused material appeared to present the large denuded areas and the platelet deposits less frequently than the non-perfused material (27).

DISCUSSION

This study shows that in the aortas of rabbits there are signs of endothelial cell injury, consisting of intracellular changes, rupture of plasma membrane, protrusion of the nuclear region, loosening of the cell from the basement membrane, and twisting of the cell. In a region of injury an endothelial cell is often disrupted close to an intercellular junction which itself is intact. Complete detachment

Fig. 11 From the lesser curvature of the aortic arch. An endothelial cell (E) is attached only by one "end" and projects into the lumen of the vessel. The cell has left behind a sharply outlined bed (arrows). 3700 \times

Fig. 12 From lateral to an intercostal artery orifice. A sharply outlined bed (B) is most likely an indication of the shedding of an endothelial cell. The edge of the region (arrows) has a double contour. 3500 \times

Fig. 13 Detail from an edge of a bed (B). The arrows point to a double contour probably representing outer and inner endothelial cell membranes. 17800 \times

Fig. 14 Area in rabbit aorta from the lesser curvature of the aortic arch. An endothelial cell bed (B) with a platelet deposited at the edge (arrow). 3500 \times

Fig. 15 Area in rabbit aorta from the lesser curvature of the aortic arch. An endothelial cell (E) projects into the lumen of the vessel. In the bed (B) an adhering platelet (PLT) is seen. 3800 \times

Fig. 16 Area of rabbit aorta from the lesser curvature of the aortic arch. Denuded areas (DA) and intact endothelial surface (ES) are seen. A platelet thrombus (T) is present on the denuded area. At the periphery of the thrombus some endothelial cells are seen to project into the lumen of the vessel (arrows). 750 \times

Fig. 17 From the lesser curvature of the aortic arch. An endothelial cell (E) with electron dense and vacuolated cytoplasm is shown. The perinuclear space of the cell is widened, and the perinuclear membrane ruptured (arrow). The nucleus has an irregular outline and condensed chromatin. The endothelial cells are loosened from the basement membrane at several sites (double arrows). The intima appears to be edematous. 8500 \times

Fig. 18 From the lesser curvature of the aortic arch. Two endothelial cells (E) appear almost completely loosened and only attached to the basement membrane (BM) by their two ends. The outer cell membrane is well separated from the basement membrane. IEM = internal elastic membrane; IJ = intercellular junction; IF = injured apparently detached endothelial cell with multiple ruptures in the peripheral cell membrane (arrows). 9400 \times

of the endothelial cells is noted, and platelet reactions occur at the site of injury.

The protrusion of the nuclear region was a prominent feature observed both by light and transmission electron microscopy. By scanning electron microscopy the nuclear protrusion appeared as a crest bulging into the lumen. Structures similar to the protruding nuclear region have been depicted and described in the scanning electron microscopic literature as "bridge like structures" first by Shimamoto *et al.* (19).

We observed a frequent association between protrusion of the nuclear region and other signs of endothelial cell injury: intracellular alterations, rupture of plasma membrane,

subendothelial blebs, and partial detachment of the cell. Like Reidy & Bowyer (16, 17) we therefore consider it likely that the cells with bulging nuclei—the "bridge like structures" of Shimamoto *et al.* (19)—are injured cells. This view is like that of Björkerud & Bondjers (2) who also found that bulging of endothelial cells preceded the disintegration and thus could be considered as an early sign of injury.

Zweifach (33) showed that pricking endothelial cells of blood capillaries with a needle caused bulging of the cells, indicating that the cells react with contraction to mechanical stress. Wajno *et al.* (13) and Joris *et al.* (9) showed that endothelial cells contain contractile material, and the question arises whether the nuclear bulging is a sign of cellular contraction in association with injury.

By scanning electron microscopy some of the elongated partly loosened endothelial cells which protruded into the vessel lumen showed a spiral-like configuration of the ends as if these cells had been twisted around their longitudinal axes. In transmission electron microscopy twisting of the peripheral parts of the cells was probably reflected by waist-like constrictions. At this stage the endothelial cell seemed to be attached to the underlayer by its two "ends." As far as we know this observation has not previously been reported.

Desquamation of endothelial cells was first indicated by the presence of partly detached single endothelial cells projecting into the vessel lumen, and by the presence of denuded intimal areas which often appeared in the form of beds left by detached single endothelial cells, as observed by Hecker *et al.* (31).

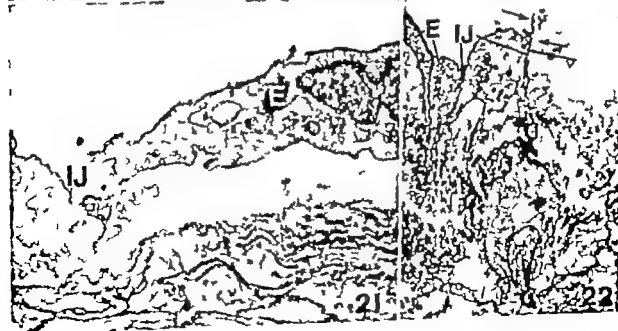
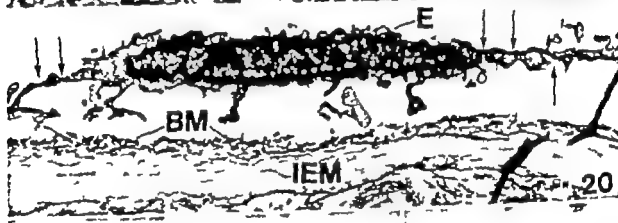
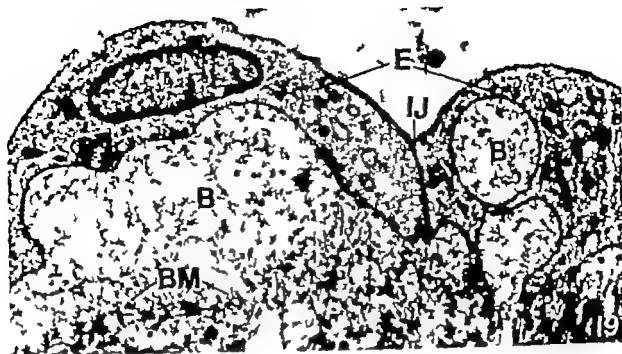
Our observations showed that intercellular junctions between endothelial cells remained intact and that cell rupture often occurred through the periphery of the cell, close to a junction. Florey *et al.* (6) showed by transmission electron microscopy that the so-called "cement lines" of silver-stained en-face endothelial preparations do not represent the junctions themselves. The silver nitrate was found to be deposited on the plasma membrane in a zone which extended beyond the

Fig 19 Area in rabbit aorta from between neighboring orifices of intercostal arteries on the same side. Endothelial cells (E) with subendothelial blebs (B) containing a granular material (edematous fluid) are shown. The cells are partly separated from the basement membrane (BM). The separation is most pronounced corresponding to the nuclear part of the cell whereas the cell appears to be well attached to the basement membrane close to the intercellular junction (IJ). 12400 \times

Fig 20 Lateral to an intercostal artery orifice. An injured endothelial cell (E) is separated from the basement membrane (BM) and attached to it only by the two ends. The peripheral parts of the cell body show several waist-like constrictions (arrows). The cytoplasm is scanty and the nucleus elongated with dense chromatin. IEM = internal elastic lamina. 10100 \times

Fig 21 Lateral to an intercostal artery orifice. An endothelial cell (E) which shows a complete disruption of the cell close to an intercellular junction. Note that a small part of cell cytoplasm (triangle) is left at the intact intercellular junction (IJ). 10500 \times

Fig 22 Area in rabbit aorta from between neighboring orifices of the intercostal arteries on the same side. E = parts of endothelial cells with intact intercellular junctions (IJ). Remnants of another endothelial cell (triangle) are seen at an intact intercellular junction. The arrow points at the two edges of the ruptured plasma membrane. These edges probably correspond to the double contour seen by scanning electron microscopy (cf. 12 and 13) at the periphery of beds from desquamated cells. 7600 \times



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junction on both sides of it. In rats treated with ϵ amino caproic acid *Ashford & Freeman* (1) found deposits of platelets and fibrin on the endothelium in femoral veins which had been subjected to a minimal trauma. Serial sections showed minute flaws of the endothelial cell plasma membrane associated with fibrin and platelets in areas close to intercellular junctions. *Shimamoto & Sunaga* (22) observed the appearance of blebs protruding from the endothelial cell surface close to the intercellular junctional areas in animals given a single oral dose of cholesterol. They also observed that platelets were often stuck to the blebs. From these studies as well as ours, it appears as if the part of the plasma membrane of endothelial cells which is in close connection with the junctional complexes constitutes a particularly "weak spot" where breaks easily occur or where dragging forces are apt to be concentrated. One possible explanation of the junction near breaks could be that the cells are more tightly fixed to the basement membrane at a junction than elsewhere. That the cell detachment was observed to originate at the centre of the cell and only rarely seemed to involve a junctional area unless complete sheets of endothelial cells were peeled off lends some support to this theory.

The question arises: what are the mechanisms of the desquamation? Does it merely reflect a random occurrence of cell death and a consequent removal of endothelium as of most other tissues, or is it a sign of vascular injury?

It right (32) and *Schwartz & Benditt* (18) showed that the endothelial cell replication was definitely accelerated in certain predilection sites of guinea pig and rat aortas. *Packham et al* (15) *Jorgensen et al* (11) and *Bjorkerud & Bondjers* (2) found signs of intimal injury with increased permeability, endothelial cell damage, and platelet reactions in aortic areas known to be predilection sites of atherosclerosis. As more fully documented elsewhere (26-27) the endothelial cell alterations described in this paper were particularly prevalent in areas of pigs probably

reflecting haemodynamic disturbances such as eddies. This would indicate that haemodynamic forces are important for the endothelial cell desquamation.

Other factors may however also be of importance. We observed that single platelets or groups of platelets adhered to altered endothelial cells or to denuded intimal surfaces. It has been suggested (10, 12-15) that the platelet reaction, occurring secondary to haemodynamic disturbances, may be the primary cause of endothelial injury. This theory is based on the knowledge 1) that platelets react in disturbed flow (15) 2) that platelets may occasionally adhere to apparently normal endothelial cells (10) and 3) that endothelial cells may be injured by platelet reactions (11). The observations in the present paper may fit in with this hypothesis, but they do not give any further evidence to support it.

Cholesterol in the diet for one or two days appeared to accelerate the endothelial cell injury (26-27). *Shimamoto et al* (20-22) reported endothelial cell alterations with increased permeability following short term cholesterol feeding. According to these authors, accelerated endothelial cell injury was noted even after a single dose of cholesterol of catecholamines.

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Fig 1 Trophoblastic cells, with multiple, pleomorphic nuclei and partly vacuolated cytoplasm, are seen in the curettage. Haematoxylin and eosin. $\times 140$



Fig 3 Trophoblastic cells are infiltrating between muscle cells in the myometrium. Inflammatory cells are also evident. Haematoxylin and eosin. $\times 320$



Fig 2 A uterine tumour in the fundic part is demarcated by arrowheads. Bar indicates 0.5 cm

are dimension of 1.8 cm was seen in the fundic part (Fig 2). Only 1.5 mm myometrium separated it from the serosal surface. Microscopically the central part of the tumour showed haemorrhage and necrosis. Large atypical cells, corresponding to those seen in curettage, were infiltrating between muscle cells in the myometrium (Fig 3) and also invaded blood vessel walls and lumens. Chronic inflammatory cells were also seen in the myometrium. Only very few mitoses were found.

No Arias-Stella phenomenon was visible any longer and the endometrial glands showed a weak proliferative tendency with partial cystic dilatation. The decidua had regressed with a dense network of reticulum fibers around the individual cells.

A corpus luteum with a cavity of 8 mm in diameter was found in one ovary having colloid-like material and calcium in the luteinized granulosa cell layer—a finding seen in the corpus luteum of pregnancy (Nelson *et al.* 1958). The theca interna zone was, however, inconspicuous. The other ovary showed fresh corpus luteum with granulosa and

TROPHOBLASTIC PSEUDOTUMOUR OF THE UTERUS

A Case Report

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Nickels J, Riseberg B & Melander S Trophoblastic pseudotumour of the uterus. A case report. Acta path. microbiol. scand. Sect. A, 86 14-16 1978.

A 52-year-old woman with vaginal bleedings had hysterectomy because curettings prompted suspicion of malignancy. A uterine tumour with deep infiltration of trophoblastic cells in the myometrium was revealed. Postoperative quantitative analysis for urinary human chorionic gonadotropin gave 1500 IU/l. The patient got no other treatment and is well 3 years after operation. The histopathological picture and the clinical course were consistent with the recently introduced diagnosis of benign trophoblastic pseudotumour which should perhaps replace the old terms "syncytioma" and "chorionepithelioma".

Key words: Uterine neoplasms, trophoblastic tumour.

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Received 1 viii 77 Accepted 1 viii 77

In 1976 Kurman *et al* reported 12 patients with localized trophoblastic invasion of the myometrium which on pathologic examination simulated a malignant tumour. The follow up studies, however, revealed a benign lesion for which the authors proposed the name "trophoblastic pseudotumour".

We also believe that this is a distinctive lesion and wish to report a thirteenth patient of exceptionally high age supporting the view of Kurman *et al* of the benign nature of the tumour.

CASE REPORT

A 52-year-old woman who had had a normal delivery at the age of 35 visited the hospital because of vaginal bleedings lasting for 18 days. The men-

strual cycles had been regular except for the last 3 months. At palpation the uterus had a size corresponding to 9-10 gestational weeks. Because two curettings at intervals of two weeks prompted suspicion of malignancy a total hysterectomy and bilateral salpingo-oophorectomy were performed.

Six days after operation the quantitative analysis for urinary human chorionic gonadotropin (HCG) gave 1500 IU/l (Pregnosticon®). Normal values for blood progesterone (0.1 ng/ml) and follicle-stimulating hormone (0.8 ng/ml) were measured 14 days postoperatively. Seven months later a pregnancy test (Pregnosticon®) was negative. No other treatment was given to the patient, and she is well three years after operation.

Histopathological findings: The curettings showed many polyhedral, trophoblastic cells with multiple, pleomorphic nuclei and abundant partly vacuolated cytoplasm (Fig. 1). The endometrial glands were in the secretory phase some showing the Arias-Stella phenomenon. Few decidual cells were seen in the stroma. No chorionic villi were found.

At operation a uterine mass with a greatest lin-

TUMOUR CELL DEATH THE PROBABLE CAUSE OF INCREASED POLYAMINE LEVELS IN PHYSIOLOGICAL FLUIDS

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Heby O & Andersson, G Tumour cell death: the probable cause of increased polyamine levels in physiological fluids. *Acta path. microbiol. scand. Sect. A*, 86 17-20, 1978.

The occurrence of extracellular polyamines in Ehrlich ascites tumour-bearing mice was studied in relation to tumour growth. The concentration of putrescine and spermidine in cell-free ascites fluid and serum was found to increase significantly with increasing tumour mass. Data is presented which suggest that the observed accumulation of extracellular polyamines is a result of a continuous release from dead or dying tumour cells. This observation is consistent with the notion that extracellular polyamines accumulate during tumour growth and following radio- and chemotherapy as a result of tumour cell death, and emphasizes the potential clinical usefulness of the polyamine analysis for evaluating tumor cell kill.

Key words: Serum polyamines, putrescine, spermidine, cell death, Ehrlich ascites tumour.

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Received 26. 77 Accepted 25. VIII. 77

The polyamines putrescine, spermidine and spermine show elevated levels in the cerebrospinal fluid (Marion *et al.* 1976), serum (Aurhøva & Romdahl 1974) and urine (Dreyfuss *et al.* 1975, Townsend *et al.* 1976, Datta *et al.* 1977) of patients suffering from cancer. Recent observations suggest that the analysis of the polyamines in physiological fluids may become a valuable tool in diagnosing cancer and in monitoring therapy. At the present time one has only speculated as to the cause of the increase in polyamine concentration in cancer patients. Thus, it has been suggested that the elevated level is both a function of growth fraction and cell death (Datt *et al.* 1977). To test this hypothesis

experimentally we have analyzed the concentration of the polyamines in cell-free ascites fluid and serum from Ehrlich ascites tumour bearing mice at various times of tumour growth. The *in vivo* growth pattern of this tumour is characterized by a continuous decrease in growth rate with increasing tumor mass (Andersson & Heby 1972). A gradual decrease in the growth fraction and a progressive increase in the duration of the cell cycle are the main determinants in growth deceleration, whereas cell loss, due to cell death and/or migration, contributes less to growth deceleration of this tumour (Tannock 1969). Thus, if the extracellular polyamine concentration is a function of the rate of multiplication there ought to be a maximal

theca interna luteinization and haemorrhage in the center

DISCUSSION

The histological picture of the heavy infiltrative tendency with necrosis and haemorrhage is typical of a trophoblastic tumour. These trophoblastic cells probably also produced the measurable urinary HCG causing the Arias-Stella phenomenon, decidual reaction and the theca interna luteinization of the corpus luteum. Unfortunately the HCG estimation was not performed until the sixth postoperative day; it was 1500 IU/l. If we assume that the half life of HCG due to renal clearance is 35 hours (Midgley & Jaffe 1968) the urinary concentration at the time of operation was about 26000 IU/L. Kurman *et al* (1976) were also able to show by the immunoenzyme method that some of the trophoblastic cells in their trophoblastic pseudotumour cases produced HCG.

The differential diagnosis from chorion carcinoma is difficult but the cell proliferation in our case was not so massive as is seen in chorion carcinoma and the mitotic activity was low. The dimorphic population of syncytio- and cytotrophoblasts often seen in chorion carcinoma was absent. The relatively long follow up period, the patient having no cytostatic treatment, also suggests a benign lesion. Conservative treatment seems to be indicated even though the published material so far is too small for definitive recommendations. The clinician must be alert in these cases because of the risk of uterine perforation by curettage.

Ewing's (1910) classification is the basis for the modern nomenclature of trophoblastic disease. His chorionadenoma destruens (invasive mole) and chorioncarcinoma are well defined and accepted entities. His term syncytial endometritis with mild infiltration of the myometrium by syncytial wandering cells is, however, unjustified and undesirable (Novak 1967; Ackerman & Rosen 1974) because it is only a normal anatomic event at the placental site after miscarriage or full term

pregnancy. This term should be abandoned.

Ewing's (1910) description of one case with a trophoblastic infiltration with more definite characteristics of a neoplasm, which he called syncytoma, has resemblances to the present trophoblastic pseudotumours (Kurman *et al* 1976). On the other hand, in 1949 Schopper and Pliess published 26 cases with deeper penetration of the uterus by trophoblastic cells than in the so-called syncytial endometritis; they introduced the term chorionepithelioma. Since then, only two additional cases with this diagnosis have been published (van Bogaert & Staquet 1977). Kurman *et al* (1976) do not mention this entity but it is possible that some of these cases, too, could be examples of trophoblastic pseudotumour. The terms syncytoma and chorionepithelioma should perhaps also be rejected and replaced with the more exact diagnosis of trophoblastic pseudotumour.

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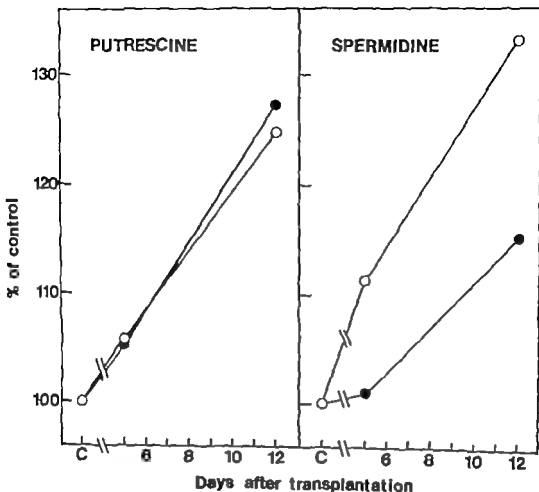


Fig. 1 Polyamine concentration in serum (O) and cell-free ascites fluid (●) of Ehrlich ascites tumour bearing mice at various times after tumour transplantation.

tion of the tumour cells. The observed accumulation of extracellular polyamines may instead be explained by a continuous release of these compounds from dead or dying cells.

Assuming that the cellular content of polyamines (Anderson & Heby 1972) is released into the ascites fluid upon tumour cell death, the specific rate of cell death which has been estimated in this study (Fig. 2) generates a total polyamine load amounting to approximately 100 nmoles/day per ml of tumour. Therefore, the potential polyamine load generated by the entire tumour is about 330 and 1150 nmoles/day at 5 and 12 days after transplantation (Fig. 3). In view of the fact

that the polyamine concentration in cell free ascites fluid only amounts to about 15 nmoles/ml, it is apparent that the major portion of the polyamines released from dead cells are eliminated from the ascites fluid and the serum. There are several possible explanations for this elimination of polyamines: 1) cellular uptake and re-utilization; 2) extra-cellular degradation; 3) urinary excretion.

A combined effect of a decreased rate of tumour cell proliferation (which may result in a decreased uptake and re-utilization of polyamines) and an imbalance between the release of polyamines and their urinary rate of excretion, may provide the basis for the

release of polyamines during the early phase of growth and a subsequent decrease with increasing tumor mass. On the other hand if the extracellular polyamine concentration is a function of tumour cell death, there ought to be an essentially constant release during the entire growth period inasmuch as the extent of cell death remains constant throughout growth.

MATERIALS AND METHODS

Polyamine analysis. Serum and cell-free ascites fluid pooled from 10-15 mice was subjected to polyamine analysis. One-ml-aliquots of serum and cell free ascites fluid were mixed with equal volumes of concentrated HCl and hydrolyzed for 14-16 hr at 110°C in teflon-sealed test tubes. The hydrolysates were adjusted to pH 11-12 with NaOH. The amines were then extracted into 2 x 2 ml of 1 butanol in the presence of 0.3 g of a salt mixture (anhydrous $\text{Na}_2\text{SO}_4/\text{Na}_3\text{PO}_4 \times 12 \text{ H}_2\text{O}$ 62.3/9.0 w/w). Following 30 min of agitation the layers were separated by centrifugation. The butanol phase was recovered acidified with 0.4 ml of 0.1 M HCl plus enough concentrated HCl to bring the pH of the butanol phase to 2 and extracted with 3 volumes (12 ml) of n hexane. The mixture was shaken heavily for 15 min. The hexane-butanol phase was discarded and the acid phase containing the polyamines was washed with diethyl ether once. Remaining ether was removed by evaporation. A 200 μ l aliquot of the extract was reacted with 1-dimethylammonaphthalene-5-sulfonyl chloride (Dns-Cl) as previously described (Heby & Anderson 1977). The Dns-amides were extracted into 500 μ l of toluene and the layers were separated by centrifugation. The toluene extract was passed through a silica gel 60 column. Subsequently a mixture of toluene ethylacetate (1:1) was used to elute the amines. The dansylated amino acids remained on the column. The eluate was evaporated to dryness and the residue dissolved in 50 μ l of toluene. Aliquots were applied to thin layer chromatography (TLC) plates and separated by one dimensional chromatography as previously described (Heby & Anderson 1977). The dansylated polyamines were quantified by means of *in situ* fluorometry utilizing an Aminco-Bowman spectrophotofluorometer equipped with a TLC-scanner and an XY recorder.

Determination of the specific rate of cell proliferation. The specific rate of cell proliferation was estimated according to a stathokinetic technique (Refrum & Berdal 1967). Tumour-bearing mice were given a single i.p. injection of vinblastine (1 mg/kg body weight) and were sacrificed at in-

tervals during a 24 hr time period. The tumour cells were collected and stained according to the Feulgen technique utilizing the fluorescent dye, 2,3-bis-4(4'-aminophenyl)-(1) = 1,3,4-oxadiazole (BAO) (Rack 1966). The number of tumour cells that had accumulated in metaphase was established for the samples obtained at 0, 2, 4 and 6 hr after vinblastine injection. The rate of entry of cells into mitosis (per cent per hr) was calculated from the steepest part of the metaphase accumulation curve. Thus the specific rate of cell proliferation ($\frac{dN}{dt}/N$) i.e. the rate of increase of cells per cell of the population, is obtained.

Determination of the rate of tumour cell death. The rate of cell death was estimated by measuring the loss of ^{125}I from mice bearing ^{125}I -deoxy-undine (^{125}I dU) labeled ascites tumour cells. Five days after transplantation the DNA of the tumour cells was labeled by two consecutive injections of 5 μCi of ^{125}I dU (specific activity 90-110 mCi/mg) with a time-interval of 20 hr. Three days after the first injection of ^{125}I dU the tumour cells were collected, washed in sterile saline and transplanted into new hosts. In order to prevent the uptake of ^{125}I by the thyroid the drinking water was supplemented with 0.1 per cent of NaI two days before the mice received the inoculum of ^{125}I dU labeled cells. Whole body ^{125}I -radioactivity was monitored by γ -counting of the individual live mice in a well-type crystal scintillator. The rate of ^{125}I -excretion from the mice was taken as an index of tumour cell death (Hofer *et al* 1969).

Chemicals. Dns-Cl was purchased from Sigma Chemical Co. St. Louis, Mo., U.S.A. thin-layer chromatography plates precoated with silica gel 60 from Merck Darmstadt, West Germany and ^{125}I dU (specific activity 90-110 mCi/mg) from the Radiochemical Center Amersham, England. BAO was purchased from Fluka Buchs, Switzerland.

RESULTS AND DISCUSSION

The present experiments show that the putrescine and spermidine concentrations in cell free ascites fluid and serum from Ehrlich ascites tumour bearing mice increases significantly during tumour growth (Fig 1). The polyamine concentrations were roughly the same in ascites fluid and serum at the various time points. The fact that there is a considerable decrease in the rate of cell proliferation from Day 5 through 12 (Fig 2) makes it highly improbable that elevated extracellular polyamine levels reflect the rate of prolifera-

SOLITARY DURAL PLASMACYTOMA

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Only four of the nine published cases of solitary extracerebral, intracranial plasmacytoma are considered authentic. In this report we describe an accidentally discovered dural plasmacytoma and draw the attention to this rare lesion which might have a different prognosis from the rather benign course of meningioma with which it is confused.

Key words. Dura, solitary plasmacytoma, extracerebral.

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Received 20. 77 Accepted 29 vii.77

Solitary extracerebral plasmacytomas are uncommon tumours which have been reported in most body organs and tissues (Dolin & Deiner 1956; Mancilla-Jimenez & Tepasoli 1976) but they are rare in intracranial structures. Mancilla-Jimenez & Tepasoli (1976) reviewed the published cases, accepted three of them as authentic and added a new case.

We report the fifth case of solitary intracranial plasmacytoma without systemic myeloma, which was accidentally diagnosed after a head injury with a fatal outcome. The differential diagnosis of this rare entity is discussed.

CASE REPORT

A 52-year-old Swedish man (case no 84/77) with no history of relevant disease fell down in January 1977 and injured the right side of his head, became immediately unconscious and was transferred to hospital. On examination a lacerated bleeding wound was found in the right temporal region; reaction to pain was poor but there were no localizing neurological manifestations. Blood tests were

within normal limits and skull X-ray revealed a fissure fracture in the right parieto-occipital region, which joined the coronary suture and proceeded down into the middle fossa. A sharply demarcated area measuring 7.0 cm in diameter was seen in the right frontal bone, with uneven thinning of bone centrally and sclerosis peripherally (Fig. 1). Echoencephalography was normal.

The patient was put under observation in the intensive care unit, where he developed cardiac and respiratory arrest from which he recovered. Craniotomy was performed on the right side because intracranial bleeding was suspected, and a large epidural haematoma and arterial bleeding were dealt with. Postoperatively the patient continued to be unconscious and carotid arteriography showed an extradural haematoma on the left side, for which a new craniotomy was performed. Laceration was seen in the left temporal lobe, and the patient died 2 days after the operation.

During the first operation the surgeon noticed an extradural tumour mass in the right frontal area, which extended to the sagittal line but had no connection with the overlying bone.

On autopsy (No 24/77) there was a small extradural haematoma in the right parietal region. An extradural pedunculated, well-circumscribed, flat, coarsely lobulated mass measuring 5 × 3 × 1 cm was found attached to the dura of the right frontal area by a short broad pedicle. The mass extended to the sagittal line but did not cross to the other side.

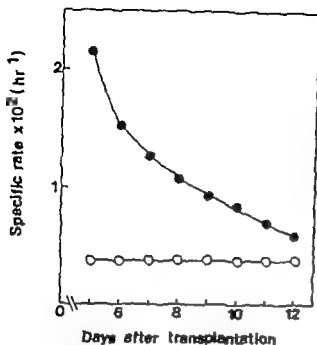


Fig 2 Specific rates of cell proliferation (●) and cell death (○) during Ehrlich ascites tumour growth.

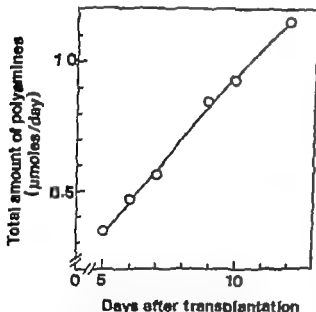


Fig 3 Theoretical release of polyamines into the extracellular fluid from dead or dying cells, as calculated from the total cellular content of polyamines (putrescine, spermidine and spermine) (Anderson & Haby 1972) and rate of cell death (Fig. 2)

observed accumulation of polyamines in ascites fluid and serum with increasing tumour mass. Thus, tumour cell death is the probable cause of increased polyamine levels in physiological fluids in relation to cancer

This conclusion is supported by the findings that chemotherapeutic treatment results in the accumulation of polyamines in cerebrospinal fluid (Morton *et al.* unpublished work) serum and urine of cancer patients (Dutrie *et al.* 1977)

This investigation was supported by the Swedish Cancer Society and the Royal Physiographical Society. A γ -counter was kindly made available for this study by the Department of Oncology at the University of Lund

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Fig. 3 Low power magnification of the tumour showing the mononuclear cellular infiltrate and the thin capillary spaces. Haematoxylin and eosin. Magnification $\times 25$.

DISCUSSION

Only nine cases of extraskeletal, intracranial solitary plasmacytoma have been previously reported. Mancilla Jimenez & Tavaroli (1976) reviewed these cases and accepted four of them as satisfying all criteria necessary for the diagnosis of extraskeletal plasmacytoma. Three cases were considered to be plasma cell granuloma and two represented intracranial lesions of multiple myeloma.

In our case the tumour was accidentally discovered during the first craniotomy and subsequently during autopsy. The patient had not suffered from any disease during his life; his death was due to the traumatic lesions and subsequent bronchopneumonia following the accident. Histological exami-

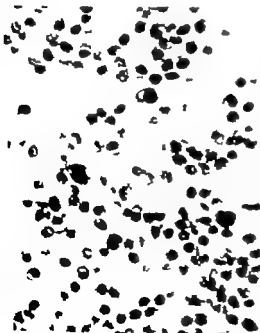


Fig. 4 The tumour cells showing eccentric cart wheel nuclei. Some cells are binucleated. Haematoxylin and eosin. Magnification $\times 300$.



Fig. 1 X-ray of the skull showing rarefaction of bone in the right parietal area with sclerosis of the borders.

or press on the brain. It fitted into a very shallow depression in the overlying frontal bone which was free from tumour tissue. The tumour was rubbery in consistency and the cut surface was homogeneously creamish white with prominent small blood vessels but no areas of necrosis (Fig. 2). This tumour was macroscopically thought to be a meningioma.

There was slight laceration of the brain tissue 3.0 cm in diameter in the right temporal lobe. A lacerated area with bleeding measuring 5.0 cm in diameter was found in the left temporal lobe and a similar area measuring 2.0 cm in diameter in the right occipital lobe. Otherwise the brain showed oedema and slight congestion. The lower lobes of both lungs were consolidated. Many small



Fig. 2 The homogenous cut surface of the tumour with prominent blood vessels and part of the tumour-free pedicle attached to the dura. Magnification $\times 3$.

shallow ulcers were seen in the stomach mucosa. The liver and spleen were of normal size and the bone marrow in the femur vertebral bodies and skull bones appeared normal macroscopically.

MATERIAL AND METHODS

Tissues were taken from the tumour mass, brain, lungs, liver, spleen, stomach, bone marrow and right frontal skull bone. Specimens were fixed in 10 per cent neutral buffered formalin, processed in routine fashion, and paraffin sections were cut at 5 μ m. Slides were stained with haematoxylin and eosin, methyl green pyronin, Congo red, methyl violet and the Gordon & Sweet silver impregnation technique was used for reticular fibres.

RESULTS

The dural tumour was rich in thin walled capillary spaces and composed almost exclusively of plasma cells and very few mature lymphocytes (Fig. 3). About 10 per cent of the plasma cell population was composed of relatively large cells which showed nuclear hyperchromatism and pleomorphism. Some of these cells were binucleated or multinucleated but no mitoses were seen (Fig. 4). The tumour was well encapsulated and the thin capsule as well as the fibrovascular pedicle was free from tumour cells. The cytoplasm of the plasma cells was pyroninophilic with the methyl green pyronin stain, but staining with the Congo red and methyl violet was negative indicating the absence of any amyloid. Silver impregnation with the Gordon & Sweet technique revealed a fine network of reticular fibres which clearly outlined the capillary spaces. No necrosis, granulation tissue or fibrosis was present in any part of the tumour.

Parenchymal haemorrhages and oedema were seen in the brain tissue. Bronchopneumonia was histologically confirmed and the stomach showed superficial shock ulcers. Osteoporotic like changes were seen in the right frontal bone which was free from tumour cells. The liver, spleen and bone marrow were not infiltrated by plasma cells and had normal structure.

AN ANALYSIS OF 38 MALIGNANT FIBROUS HISTIOCYTOMAS IN THE EXTREMITIES

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Eklöf, T O & Rantakokko, V. An analysis of 38 malignant histiocytomas in the extremities. Acta path. microbiol. scand. Sect. A, 86 25-35 1978.

All the malignant soft tissue tumours in the extremities and limb girdles reported to the Finnish Cancer Registry between 1960-1969 were reviewed. From a total of 246 sarcomas, 38 were diagnosed as malignant fibrous histiocytoma. There was an equal number of male and female patients with the median age of 67 years. The thigh was the most frequent site and the majority of the tumours originated in the deep soft tissues. The predominant treatment was excision followed by radiation therapy. In 17 patients there were one or more recurrences and in 21 patients there was a metastatic spread ascertained by biopsy, autopsy or clinical or radiographic evidence. There were 11 survivors with a minimum of 5 years' follow-up; seven patients died of an intercurrent disease and the remaining 20 patients were considered victims of their tumour. The findings that seemed to favour a poor prognosis were higher age and female sex of the patient as well as deep location, large size, necrotic areas, and high mitotic activity of the tumour.

Key words: Soft tissue tumour; fibrous histiocytoma, malignant; fibrosarcoma, malignant; atypical fibrosarcomatous.

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Received 10.11.77 Accepted 10.11.77

Among the pleomorphic soft tissue sarcomas, malignant fibrous histiocytoma (malignant fibrosarcoma, fibrosarcomatous) is probably the least known. The published series include those of O'Brien & Stout (1964), Kempson & Kyriakos (1972) and Soule & Enriquez (1972). Mackenzie reviewed the topic in 1975. In a recent survey of malignant soft tissue tumours in the extremities and limb girdles in Finland 1960-1969, 38 of the total of 246 sarcomas were diagnosed as malignant fibrous histiocytoma.

The pertinent clinical and pathological data were analyzed, and are reported in the present paper.

MATERIAL AND METHODS

The original material consisted of the malignant soft tissue tumours in the extremities and limb girdles reported to the Finnish Cancer Registry during the decennium 1960-1969. There were 409 occurrences altogether. The paraffin blocks or original slides were requested from pathological institutions for review. They were available in 366 cases. From representative blocks, sections were stained

nation did not reveal plasma cell infiltration except in the right frontal dural mass which led to rarefaction of the overlying bone, probably due to long-standing pressure. Protein electrophoresis was not performed because of the rapid fatal outcome.

The preliminary clinical and macroscopical pathological diagnosis was that of a meningioma. The location of the tumour its lobulated flattened appearance, rubbery consistency and lack of pressure on the brain are common features of both meningioma and meningeal plasmacytoma. Histological examination of such tumours is the only way to achieve the correct diagnosis, and the mere existence of this entity makes microscopic examination of all tumours thought to be a meningioma mandatory. The reticular fibre pattern was similar to that of angio-blastic meningioma but different from that seen in meningiotheliomatous meningioma. In the latter reticular fibres divide the tumour into distinct lobules (Rubinstein 1972).

The conditions which should be considered in the differential diagnosis of intracranial plasmacytoma microscopically are plasma cell granuloma and a local lesion in a case of multiple myeloma. Furthermore, a long period of follow up of the patients is necessary to exclude the possibility of transformation of a solitary intracranial tumour into a disseminated myeloma. In authentic cases of solitary meningeal plasmacytoma no abnormal proteins are present in the serum or

urine, and bone marrow specimens and bone surveys should be normal. The histological picture of plasma cell granuloma is characterized by the presence of an admixture of plasma cells, lymphocytes and macrophages on a background of partly necrotic tissue including areas of fibrosis and others of granulation tissue.

The proper diagnosis of intracranial plasmacytoma is essential for the prognosis and management of the patients. Two of the four authentic cases of plasmacytoma have been followed for 4.5 and 50 years without recurrence or transformation into multiple myeloma. The case reported by Mancilla Jimenez & Tarassoli recurred locally after 5 years despite complete surgical excision and irradiation to the tumour area.

The authors wish to thank Mrs Siv Wiklund and Miss Christina Andersson for secretarial help, and Mr Frank Butkowski for photography.

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with haematoxylin-van Gieson, haematoxylin and eosin, Gomori reticulin PAS with and without diastase Alcian blue at pH 2.5 Mason trichrome, azan, PTAH toluidine blue Fontana-Masson silver stain, and Prussian blue reaction according to standard techniques.

When lymphomas, melanomas, metastatic carcinomas, tumours thought to have originated in bone and benign lesions were excluded, there remained 246 cases which were considered malignant soft tissue tumours. From these 38 were diagnosed as malignant fibrous histiocytoma by using the histological criteria described in the papers listed in the introduction. In two cases the original slides only were available.

When counting mitoses, the most cellular areas were chosen. A Leitz Dialux microscope with a NM 40/0.65 objective was used. The area of the high power field (HPF) was 0.16 mm².

The hospital files were also reviewed and analysed. None of the patients were lost to follow up.

The significance of the findings were tested by using the Fisher exact probability test.

RESULTS

Clinical findings The case histories of diagnosed malignant fibrous histiocytomas are summarized in Table 1. There was an equal number of male and female patients. The median age was 67 years, with a range from 30 to 87 years.

The thigh (including hip) was the most common site with 45 per cent of the tumours. 27 (71 per cent) were in the lower limb. In the upper arm (including shoulder) there were four tumours and six were in the lower arm. No tumours were in the foot and only one was in the hand. The majority (60 per cent) of the tumours were considered to have originated in the deep soft tissues (inside the muscular fascia). A third of the tumours was fixed to or ulcerated the skin.

The diameter of the tumours varied from 3 to 18 cm. The median size was 8 cm.

The usual clinical complaint was a growing mass. Only one patient stated a preceding trauma to the tumour site, seven years earlier. The median duration of symptoms before the diagnosis was six months (range one month to 12 years).

Two tumours were considered inoperable, and only radiation therapy was given. Two

were enucleated and in two cases there was a primary amputation. In others, a local excision was performed in eight of them. Secondary amputations after one or more recurrences were performed in four cases. Radiation therapy was given to 24 patients at some stage of their disease usually post-operatively.

In 17 cases (45 per cent) there were one or more recurrences. A metastatic spread was found in 21 cases (55 per cent). This could be confirmed histologically by biopsy or at autopsy in seven cases. There was radiographic evidence in all the patients with pulmonary metastases.

There were 11 survivors (5 to 12 years after the diagnosis); seven patients died of an intercurrent disease and in 20 patients (53 per cent) the tumour was considered to be the cause of death. Almost all (18) of them died within 2.5 years, the remaining two living 6 and 8.5 years, respectively.

Macroscopical appearance The tumours were usually infiltrating into the surrounding tissue. No remarkable macroscopic features were noticed, but the descriptions were frequently unsatisfactory.

Microscopical appearance The wide histological spectrum these tumours can show is illustrated in Figs. 1-5. Some histological

Fig 1 Malignant fibrous histiocytoma showing a storiform pattern with atypical, spindle fibroblastic cells. Two giant cells with peripherally arranged nuclei are seen in the centre. Haematoxylin-van Gieson. $\times 90$

Fig 2 Patternless area with bizarre histiocytic cells in a malignant fibrous histiocytoma. Moderate inflammatory cell infiltration is seen on the back ground. Haematoxylin-van Gieson. $\times 225$

Fig 3 Myxoid area in a malignant fibrous histiocytoma. The connective tissue septum contains mononuclear inflammatory cells. Haematoxylin-van Gieson. $\times 90$

Fig 4 Pericytomatous pattern with many thin-walled blood vessels in a malignant fibrous histiocytoma. The pleomorphism of the cells is greater than that in hemangiopericytoma. Haematoxylin-van Gieson. $\times 90$



TABLE 1 Summary of Case Histories of the

Case	Sex	Age	Location S = superfl., D = deep	Duration of symptoms (months)	Diameter (cm)	Primary operation
Survivors						
1	M	82	Thigh D	36	10	Excision
2	M	40	Thigh D	2	8	Excision
3	F	54	Lower arm S	5	5	Excision
4	M	73	Thigh D	7	9	Excision
5	M	64	Leg S	4	4	Wide exc.
6	F	76	Upper arm S	5	7	Excision
7	M	36	Leg S	11	6	Wide exc.
8	M	47	Upper arm D	12	5	Excision
9	F	67	Thigh S	15	7	Excision
10	F	69	Leg S	24	5	Excision
11	F	47	Thigh D	12	7	Wide exc.
Dead of an intercurrent disease						
12	M	77	Upper arm S	8	13	Biopsy
13	M	63	Thigh S	12	5	Wide exc.
14	M	87	Leg S	7	4	Excision
15	F	48	Leg D	8	5	Excision
16	M	59	Lower arm S	7	8	Wide exc.
17	M	73	Leg D	8	14	Wide exc.
18	M	6	Thigh D	12	15	Excision
Dead of the tumour						
19	M	70	Thigh D	6	11	Excision
20	F	62	Leg D	7	18	Biopsy
21	M	49	Thigh D	2	6	Enucleation
22	F	74	Lower arm D	8	18	Amputation
23	F	75	Leg D	6	16	Excision
24	M	80	Upper arm D	18	8	Excision
25	F	66	Thigh D	24	7	Excision
26	F	76	Thigh D	10	17	Excision
27	F	42	Hand D	10	3	Excision
28	F	72	Thigh D	1	7	Excision
29	M	72	Lower arm S	6	9	Excision
30	F	49	Thigh D	4	12	Excision
31	M	38	Thigh D	84	8	Excision
32	M	48	Thigh D	8	14	Wide exc.
33	F	73	Lower arm S	11	5	Excision
34	F	80	Leg D	2	8	Excision
35	F	71	Leg S	3	4	Enucleation
36	F	70	Thigh D	11	8	Excision
37	M	65	Lower arm S	144	10	Wide exc.
38	F	56	Leg S	11	4	Amputation

features of the tumours are listed in Table 2. The basic structure varied from storiform to fasciculated, whorled and patternless. The amount of collagen was highly variable even within one and the same section. The reticulin network was dense, but small areas

could be found with cell clusters free of fibrils (Fig. 8). Small myxoid areas were found in 12 tumours, and in two tumours they were moderately large (Fig. 9). The vascularity was variable, too, but usually rich. Sinusoidal thin-walled blood vessels were

Radiation therapy	Number of recurrences	Sites of metastasis H = histological confirmation	Follow-up time (years) and other remarks
+	H		5
+	1	Node H	6 sec. amputation
+	3		7
+	4		8 sec. amputation
—	5		8
—	5		8
—	0		10
+	2		10
—	0		12
—	1		12
+	1		12
Time (months) and cause of death			
+	0		6 cardiac insufficiency
—	0		6 cardiac condition
+	0		6 pneumonia
+	0		18 suicide
—	0		26 brain insult, autopsy: no tumour
—	4		36 cardiac insuff., autopsy: no tumour
+	0		34 uremia, autopsy: no tumour
Time of death (months): other remarks			
—	0	Lung	1
+	0	Disseminated	4 autopsy: metastatic tumour
+	1	Lung	5
—	0	Lung	6
—	0	Lung	6
+	0	Lung, soft tissue	7
+	0	Lung, node	12
+	3	Soft tissue	12
—	1	Lung H	12
+	0	Lung	16
+	1	Lung	17 sec. amputation
+	1	Lung	18
+	1	Lung, node	24
+	8	Disseminated	24 autopsy: metastatic tumour
—	3	Lung	26
+	0	Disseminated H	30
+	2	Lung	30 sec. amputation
—	2	Lung	31
+	0	Lung H	32
+	4	Disseminated H	102

locally a frequent finding (Fig. 4) Haemorrhages were seen occasionally and haemoderlin was identified in six tumours

The tumours were highly cellular. The basic stromal cell varied from a spindle fibroblastic cell to a plumper and larger

histiocytic cell with a vesicular nucleus and a prominent nucleolus (Fig. 1 and 2). Atypical mono- or multinucleated giant cells were found in almost all cases. The nuclei were arranged in peripheral cytoplasm reminiscent of Langhans-type giant cells in 20

TABLE 1 Summary of Case Histories of the

Case	Sex	Age	Location S = superf D = deep	Duration of symptoms (months)	Diameter (cm)	Primary operation
Survivors						
1	M	82	Thigh D	36	10	Excision
2	M	40	Thigh D	2	8	Excision
3	F	54	Lower arm S	5	5	Excision
4	M	75	Thigh D	2	9	Excision
5	M	64	Leg S	4	4	Wide exc.
6	F	76	Upper arm S	5	?	Excision
7	M	36	Leg S	6	6	Wide exc.
8	M	47	Upper arm D	12	5	Excision
9	F	67	Thigh S	15	7	Excision
10	F	69	Leg S	24	5	Excision
11	F	47	Thigh D	12	?	Wide exc.
Dead of an intercurrent disease						
12	M	77	Upper arm S	6	13	Biopsy
13	M	63	Thigh S	12	5	Wide exc.
14	M	87	Leg S	?	4	Excision
15	F	48	Leg D	8	5	Excision
16	M	39	Lower arm S	?	8	Wide exc.
17	M	73	Leg D	6	14	Wide exc.
18	M	76	Thigh D	12	15	Excision
Dead of the tumour						
19	M	70	Thigh D	6	11	Excision
20	F	62	Leg D	7	18	Biopsy
21	M	49	Thigh D	2	6	Enucleation
22	F	74	Lower arm D	8	18	Amputation
23	F	75	Leg D	6	16	Excision
24	M	80	Upper arm D	18	8	Excision
25	F	66	Thigh D	24	7	Excision
26	F	76	Thigh D	10	17	Excision
27	F	42	Hand D	10	3	Excision
28	F	72	Thigh D	1	?	Excision
29	M	72	Lower arm S	8	9	Excision
30	F	49	Thigh D	4	12	Excision
31	M	38	Thigh D	84	8	Excision
32	M	48	Thigh D	6	14	Wide exc.
33	F	73	Lower arm S	6	5	Excision
34	F	80	Leg D	2	8	Excision
35	F	71	Leg S	3	4	Enucleation
36	F	70	Thigh D	8	8	Excision
37	M	65	Lower arm S	144	10	Wide exc.
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features of the tumours are listed in Table 2. The basic structure varied from storiform to fasciculated whorled and patternless. The amount of collagen was highly variable even within one and the same section. The reticulin network was dense but small areas

could be found with cell clusters free of fibrils (Fig. 8). Small myxoid areas were found in 12 tumours, and in two tumours they were moderately large (Fig. 3). The vascularity was variable too, but usually rich. Sinusoidal thin-walled blood vessels were

Fig. 5. Malignant fibrous histiocytoma containing plump histiocyte cells. Nuclei are vacuolar and nucleoli are prominent. The binucleated cell in the centre field resembles a Reed-Sternberg cell. Haematoxylin-van Gieson $\times 225$.

Fig. 6. Massive inflammatory cell phagocytosis shown by a large cell. To the right of that, an atypical mitotic figure is seen. Haematoxylin-van Gieson. $\times 360$.

Fig. 7. A large, bizarre giant cell with a wispy cytoplasm containing innumerable round hyaline globules. PAS with diastase $\times 360$.

Fig. 8. The reticular pattern of malignant fibrous histiocytoma is dense but in the upper and right parts of the figure there are small cell clusters free of fibrils. Gomori reticulum. $\times 225$.

tumours (Fig. 1). Cells with two to four nuclei with prominent nucleoli, thus resembling Reed-Sternberg cells, were also a frequent finding (Fig. 5). The cytoplasm was usually large sometimes vacuolated or wispy. In 29 tumours cell phagocytosis was seen (Fig. 6) and in 15 cases giant cells contained round hyaline globules which stained positively with PAS and were resistant to prior treatment with diastase (Fig. 7). Giant cells of osteoclast-type or of classical Touton-type were demonstrated in few tumours. Xanthoma cells were found in ten instances, usually at the margins of the tumour nodules.

Mast cells were moderately or highly numerous in 14 cases. Inflammatory cells were a common finding also apart from necrotic areas. In 23 cases there was a moderate or dense diffuse infiltration of lymphocytes (Fig. 1).

A similar infiltration of polymorphonuclear leukocytes was found in eight tumours (Fig. 5). The number of eosinophils and plasma cells was usually low.

The average mitotic frequency varied from 6 to 71 per ten high power fields, the average being 24. Pathological mitotic figures were seen in all the tumours (Fig. 6). Necrotic areas were found in 23 tumours, 12 of which were extensive.

The effect of clinical and pathological pa-

rameters on the prognosis In the following chapter the survivor and dead-of-tumour groups are compared to each other. There were about twice as many female as male patients dying of the tumour. The victims of the tumours were on an average 5 years older than the survivors. The anatomic distribution of the tumours was practically similar in both groups. The deep tumours seemed to indicate a poorer prognosis, 75 per cent of the patients dying of their tumour where as only 45 per cent of the patients with a superficial tumour died.

The average diameter of the tumours was 50 per cent larger in the patients who died of the disease than in those who survived. The duration of the symptoms before the diagnosis was not different. The mode of treatment was not different, either but it may be of interest that both patients with a primary amputation died of the tumour. In one of the cases (38), the amputation level was too low as evidenced by the recurrences in the leg stump. The number of recurrences was equal in both groups.

Necrotic areas were found in 36 per cent in the survivor group and in 80 per cent of the patients with a fatal outcome.

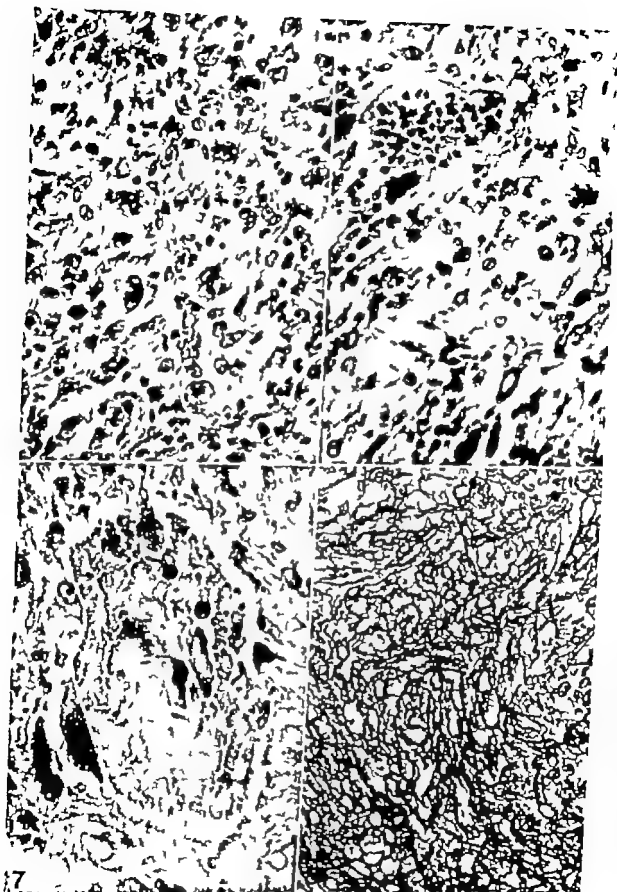
The degree of atypia did not correlate with the prognosis, neither did the occurrence of myxoid areas, mast cells, inflammatory cells, xanthoma cells, or different types of giant cells.

The average number of mitoses per ten high power fields differed only by five (26 vs. 21) but in the dead-of-tumour group 60 per cent of the tumours showed more than 20 mitoses, whereas the respective percentage in the survivor group was only 27 per cent.

The significances of all the differences mentioned above were tested statistically. Only the occurrence of necrotic areas almost significantly ($p < 0.05$) indicated a poor prognosis.

DISCUSSION

The diagnosis of malignant fibrous histiocytoma was based on the overall structure,



often suggestively storiform, the presence of fibroblastic cells, and the infiltration of inflammatory cells. The variability of the histology has been emphasized by other authors (Kempson & Kyrleas 1972; Mackenzie 1973) and this feature was conspicuous in this series, too.

Owing to the pleomorphism and high mitotic activity there did not seem to be much difficulty in considering these tumours malignant, which is in contrast to some earlier reports (O'Brien & Stout 1964; Kyrleas & Kempson 1976). Only one of the tumours was originally diagnosed as benign (27). Nevertheless, the differential diagnosis of superficial malignant fibrous histiocytoma and atypical fibrous histiocytoma of the skin may be very difficult. The latter tumours were considered to occur exclusively in the sun-damaged atrophic skin of elderly people (Kempson & McGowan 1964) but in later studies these tumours have been found also in the trunk and extremities of younger persons (Fietan & Hebing 1973; Dahl 1976). There may be no certain criteria for distinguishing these tumours from their malignant counterparts. Atypical fibrous histiocytomas are, however usually but not without exception, small tumours (less than 2 cm in diameter) and confined to the skin or superficial subcutaneous tissue (Dahl 1976). Histologically they may lack the stromal cell anaplasia found in malignant fibrous histiocytomas (Soule & Enriques 1972). The prognosis is quite different, although, according to Dahl (1976) there is evidence of metastasis in at least five cases of atypical fibrous histiocytoma.

Another serious difficulty is to distinguish malignant fibrous histiocytoma from other pleomorphic sarcomas: liposarcoma, rhabdomyosarcoma, and myxofibrosarcoma, a new entity described recently by Angerall *et al.* (1977). Pleomorphic liposarcoma should contain inequifocal multinucleated or signet ring lipoplasts, which were not observed in the present series. The criteria of pleomorphic rhabdomyosarcoma are to some extent disputable but if the presence of cross stri-

ations is required none of the tumours could be diagnosed as rhabdomyosarcoma. In PTAH preparations a slight staining in the cytoplasm could sometimes be discerned. This may correspond to the filaments described in the ultrastructural study by Fu *et al.* (1973). A few tumours showing larger myxoid areas may fulfil the criteria of myxofibrosarcoma of Angerall *et al.* (1977). These authors, however classified myxofibrosarcoma as a type of fibrohistiocytic tumour and it is thus understandable that there is some overlapping between malignant fibrous histiocytoma and myxofibrosarcoma.

Enranger & Smith (1976) paid attention to the fact that it is sometimes difficult to distinguish hemangiopericytoma from fibrous histiocytoma. In the present study however there were no problems in this respect. Although the vascularity was, at least focally rich, the histology was clearly different from that of hemangiopericytoma.

Kyrleas & Kempson (1976) described seven cases of the tumour they designated as inflammatory fibrous histiocytoma because of the heavy infiltration of inflammatory cells, especially polymorphonuclear leukocytes. All the tumours behaved in a malignant fashion. In the present study eight tumours were found with a marked infiltration of polymorphonuclear leukocytes. In our opinion there seems to be no reason why these tumours should be separated from others, as the microscopical appearance differs in no other way and the prognosis is identical with the rest of the tumours.

An interesting finding was the existence of PAS positive diastase resistant hyaline globules resembling Russell bodies in some tumour giant cells. Kindblom *et al.* (1975) described similar globules in pleomorphic liposarcoma. Srenston *et al.* (1975) have mentioned them in osteosarcoma, and we have observed globules in a few malignant giant cell tumours of soft tissues. This shows that hyaline globules are not specific for any malignant mesenchymal tumour.

The finding that the thigh is the most common site of malignant fibrous histio-

TABLE 2. *Some Histological Features of Malignant Fibrous Histiocytoma*

Case	Necrosis	Number of mitoses (per 10 HPF)	Myxoid areas	Giant cells	Mast cells	Inflammatory cells
Survivors						
1	—	17	—	++	+++	++
2	+++	15	—	—	+	+
3	—	35	—	++	+	+
4	—	7	++	++	+	++
5	+	15	+	++	++	+
6	—	27	—	+++	++	+++
7	—	71	—	++	—	—
8	+	15	—	+	+	—
9	—	18	+	++	+++	++
10	+++	9	+	++	—	++
11	—	6	+	+++	+++	+++
Dead of an intercurrent disease						
12	—	56	—	+	+	++
13	++	12	—	++	+	+
14	—	12	—	+	+++	++
15	—	9	—	+	+++	+
16	—	8	—	+	+++	+++
17	+	15	—	+++	+	+
18	+++	17	+	++	+	++
Dead of the tumour						
19	++	35	+	+	++	+
20	+++	30	—	+	+	+
21	+++	22	—	+	+	++
22	+	64	—	+++	—	+
23	—	9	—	+++	++	++
24	+	32	+	+	—	+
25	++	24	—	+	—	+++
26	+	50	—	+	+	++
27	+	25	—	+	+	++
28	+++	35	—	++	+	+
29	+++	25	+	+	+	+
30	—	10	—	+	?	+
31	+	8	—	+	++	+++
32	—	10	—	++	+	++
33	+	58	++	+	+	+
34	+	10	+	+++	+	++
35	++	40	—	++	++	++
36	++	12	—	+++	++	++
37	—	15	+	++	?	++
38	+	11	+	—	++	+

Key — = none
 + = a few
 ++ = a moderate amount
 +++ = a remarkable amount

14 *Sokal E. H. & Enriquez P.* Atypical fibrous histiocytoma, malignant fibrous histiocytoma, and epithelioid sarcoma. A comparative study of 65 tumours. *Cancer* 30: 128-143 1972

15 *Salt H. D., Russell W. O. & Martin R. G.* Sarcoma of soft tissue: Clinical and histopathologic parameters and response to treatment. *Cancer* 35: 1478-1483 1975

cytoma is in accordance with previous reports. There was, however no male predominance, and the average age of the patients was higher than that in earlier series (Mackenzie 1975).

The treatment consisted of excision frequently followed by radiation therapy. The number of recurrences suggests that the primary operation was not usually adequately wide. The effect of the radiation therapy is difficult to evaluate retrospectively but at least we can state that no dramatic cures were obtained.

The prognosis was rather poor 71 per cent of the patients dying during the follow up period, some of them, however obviously from other causes than their tumour. The fatal outcome of the disease was favoured by higher age and female sex of the patient as well as deep location, size over 8 cm, necrotic areas and mitotic frequency more than 20/10 HPF of the tumour. Owing to the small number of patients from these parameters only necrosis was shown to correlate almost significantly with a bad prognosis.

Only five patients were autopsied and the cause of death of the majority of the patients was determined on the basis of the clinical and especially radiographic evidence. There remains of course the possibility of a co-existing visceral neoplasm but such a coincidence would presumably be rare.

Finally we wish to pay attention to one additional fact according to the results of this survey malignant fibrous histiocytoma is a remarkably more common tumour than previously believed (O'Brien & Stout 1964). In two recent but considerably smaller series the proportion of malignant fibrous histiocytomas has been comparable to that of ours (Sait *et al* 1975 and Simon & Enneking 1976).

Most of the sectioning and staining procedures were performed by Mrs. Anja Ikonen whose great expertise is acknowledged.

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We owe our sincere thanks to the directors of all pathological institutions in Finland for placing the histological material at our disposal. Our thanks are also due to the heads of hospitals who permitted the review of clinical data.

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CARCINOMA OF THE UTERINE CERVIX AND DYSPLASIA IN GREENLAND

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Nielsen, N. Højgaard Mikkelsen, F. & Hansen, J. P. Hart. Carcinoma of the uterine cervix and dysplasia in Greenland. *Acta path. microbiol. scand. Sect. A* 86: 36-44, 1978.

A preliminary study based on officially recorded cases of cervical carcinoma, dysplasia and carcinoma in situ in Greenlandic women over the period 1955-1975 revealed a total of 117 cases of carcinoma and 119 cases of dysplasia and carcinoma in situ. The incidence of invasive carcinoma age-adjusted to "European standard population" (Doll 1976) exhibited a rise from 20.9 per 100 000 in 1955-1959 to 34.8 in 1970-1975. The latter incidence rate is one of the highest in the world and about 2.5 times as high as the most recently recorded incidence in Denmark. Age-specific incidence rates for cervical carcinoma in Greenland for 1970-1975 showed significantly higher values in the age groups 15-49 and 60-64 years as compared with the most recent Danish rates. The crude incidence of dysplasia and carcinoma in situ per 100 000 women over 15 years of age rose from 10.5 for 1955-1959 to 137.5 for 1970-1975. The corresponding incidence for severe dysplasia and carcinoma in situ was 93.7 for 1970-1975. There has most likely been a more complete registration of these initial stages during recent years although organized detection work has not been initiated.

Key words: Cervical cancer, eskimo, Greenland.

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Received 18 vii 77 Accepted 13 ix 77

Since 1943 the incidence of carcinoma of the uterine cervix in Denmark has been thoroughly investigated (Clemmesen 1974, 1976). Although administratively a part of Denmark, Greenland has not been included in the Danish incidence studies. The Greenlandic community differs from the Danish *inter alia* in socio-economic status (Ministry for Greenland 1976) and as regards the prevailing pattern of sexual practice (Olsen 1974), both these factors being known to affect the epidemiology of cancer of the cervix (Rotkin 1967, Stamler *et al.* 1967). It would be of interest, therefore, to clarify the

incidence in Greenland and to make a comparison with conditions in Denmark.

The present study based on officially registered cases during the period 1955-1975 elucidates for the first time the incidence in Greenland of invasive cervical carcinoma as well as of dysplasia and carcinoma in situ. A comparison has been made with the corresponding incidence in Denmark.

Population and medical facilities. In 1953 Greenland which till then had been a Danish colony, became a part of the Danish Kingdom. Ethnically the native Greenlandic population is an Eskimo-Caucasian mixture, the Caucasian admixture being on an average

25-30 per cent (Kusmeyer-Nielsen *et al* 1971 Persson 1970) From 1955 to 1975 this population group increased from 23,000 to 40,000 which means that about 40 per cent today are under 15 years of age. There is an equal number of either sex. The purely Danish group of the population, mainly men, increased over the same period from 1700 to 8000.

Greenland is divided into 16 medical districts, each with a hospital situated in a central town of the district. Already in 1955 55 per cent of the population lived in these district towns, and in 1975 this percentage had increased to 75 (Ministry for Greenland 1976). All medical service is free of charge. The only hospital with separate surgical and medical wards is "Dronning Ingrid's Hospital" (Queen Ingrid's Hospital) in the largest town of Greenland, Godthåb. This hospital has acted as a referral centre for the whole of Greenland since 1957. Furthermore, a number of patients have been transferred to treatment centres in Copenhagen since the establishment in 1954 of a regular air service between Denmark and Greenland. Most patients with suspected or verified malignant

diseases are transferred either to "Dronning Ingrid's Hospital" or to Denmark.

MATERIAL AND METHODS

All histological and cytological investigations of samples from Greenland during the period 1955-1975 have been reviewed. From 1955 to 1958 the investigations were carried out by Dr B. Vinstrup, Pathologist-in-Chief Bispebjerg Hospital, Copenhagen, from 1959 to 1970 by Dr K. Schourop, Pathologist-in-Chief Copenhagen County Hospital in Glostrup, and since June 1970 at Rigshospitalet, the University Hospital in Copenhagen. Further more, all death certificates from Greenland, the data from Greenland recorded at the Danish Cancer Registry and the files of the Ministry for Greenland concerning patients transferred to treatment centres in Denmark were reviewed. Death certificates were introduced in Greenland on 1st January 1967. Reports to the Danish Cancer Registry are available from 1957 onwards although, up to 1963 reporting was not very consistent. Only since 1963 has notification been fairly regular although still too inconsistent for publication. It was necessary to obtain supplementary data from hospital records. Consequently the case notes for all patients admitted to the surgical ward, "Dronning Ingrid's Hospital" up to and including 1975 have been reviewed.

TABLE 1 Greenland 1955-1975 Distribution by Age of Women Born in Greenland (Ministry for Greenland Copenhagen)

Age	1955-1959 (mean)	1960-1964 (mean)	1965-1969 (mean)	1970-1974 (mean)	1975 1 L
0-14	5935	7372	8957	8947	8127
15-19	1365	1568	1488	1908	2175
20-24	1150	1275	1523	1413	1658
25-29	1045	1119	1151	1525	1177
30-34	825	1053	1064	1135	1248
35-39	673	744	1019	1046	1013
40-44	600	659	758	1001	987
45-49	550	579	616	719	891
50-54	440	518	563	595	595
55-59	360	409	491	530	524
60-64	263	340	380	435	441
65-69			296	312	377
70	370	436	284	426	491
Total	13380	16030	18368	19842	19702

TABLE 2. *Incidence Cervical Carcinoma Greenland 1955-1975 Number of Cases by Age Diagnosed in Indigenous Greenland Women Crude Annual Rates per 100,000 Females over 15 Years of Age Age-adjusted Rates for all Age Groups ("European Standard Population")*

Age	1955-1959	1960-1964	1965-1969	1970-1974	1975
15-19	—	—	—	1	—
20-24	—	—	1	3	—
25-29	1	2	4	3	2
30-34	4	2	4	5	1
35-39	—	3	4	10	3
40-44	3	4	3	9	2
45-49	1	3	1	5	4
50-54	1	1	4	3	1
55-59	—	3	2	—	1
60-64	—	—	2	4	3
65-69	—	1	—	1	—
70+	—	—	—	1	1
Total	10	19	25	45	18
Crude rate (over 15)	26.2	43.8	33.1	82.6	155.5
Age-adjusted rate (all age groups)	20.9	43.4	44.8	70.3	143.9

Vital statistical data including the average age distribution of indigenous Greenland women during the five-year registration periods and the age distribution as at 1st January 1975 (Table 1) were obtained from the Statistical Office, Ministry for Greenland Copenhagen. Age-specific incidence rates of cervical cancer in Denmark, 1968-1972 were made available by the Danish Cancer Registry. Each case was registered by date of diagnosis, name, date of birth, place of residence and race only indigenous Greenland women being included. The 21 year period of registration was divided into four 5 year periods 1955-1959 1960-1964 1965-1969 1970-1974 and a single year 1975. The incidence rate is given as the average annual number of cases diagnosed per 100 000 women. Although the number of cases in each age group was very low the incidence rates for cervical cancer were age-adjusted to "European standard population" (Doll 1976).

In order to illustrate the difference between the incidence of cervical cancer in Greenland and in Denmark, an indirect method was used, applying the Danish age-specific rates (Clemmensen 1964 1969 1974) to calculate the expected number of cases of cervical carcinoma in Greenland, assuming the incidence in the Greenlandic population at any time to be the same as in Denmark. The most recent age-specific rates for Denmark, 1968-1972 were used as hypothetical rates up to 1975.

To obtain a greater number of cases in each age group the 6-year period 1970-1975 was applied

In order to clarify the latest age-specific incidence rates. For this period cases of severe dysplasia and carcinoma in situ were classified in a special group to facilitate comparison with corresponding age-specific rates of invasive cervical cancer in Denmark. The Mantel-Haenszel test (Ipsen & Frig 1970) was used for statistical evaluation of the observed/expected ratio. Otherwise 95 per cent confidence limit factors for estimates of Poisson-distributed variables were used according to Harsanyi *et al* (1962).

RESULTS

In the period 1955-1975 117 cases of cervical cancer and 119 cases of dysplasia or carcinoma in situ were diagnosed (Tables 2 and 3). One of the patients is included in both groups, because she developed invasive carcinoma 4 years after conization for severe dysplasia.

Table 4 gives data concerning all tissue specimens sent from Greenland to Denmark for histological examination during the years 1955-1975. The average annual number of all types of biopsy specimens per 1000 adults of both sexes over 15 years of age increased from 10.2 in 1955-1959 to 26.4 in 1960-

TABLE 3 *Dysplasia and Carcinoma in Situ of the Uterine Cervix: Greenland 1955-1975. Number of Cases by Age Diagnosed in Indigenous Greenland Women. Crude Annual Rate per 100,000 Females over 15 Years of Age*

Age	1955-1959	1960-1964	1965-1969	1970-1974	1975
15-19	-	-	-	2	2
20-24	-	-	2	3	1
25-29	-	2	2	11	8
30-34	-	1	4	13	4
35-39	3	2	2	10	3
40-44	1	-	1	19	2
45-49	-	1	3	8	-
50-54	-	1	1	1	-
55-59	-	-	1	2	-
60-64	-	1	-	-	-
65-69	-	-	-	1	-
70+	-	-	-	-	-
Total	4	8	16	71	20
Crude rate (over 15)	10.3	14.4	34.0	130.3	172.8

1964. However during the last 11 years, 1965-1975 the number has been almost constant. The proportion of cervical biopsies was about 20 per cent in 1955-1969 but has increased during the last 6 years up to 28 per cent, corresponding to an average of

24.2 cervical biopsies annually per 1000 women over 15 years of age. The maximum number of cervical biopsies in a single year (363) was taken in 1971.

Invasive carcinoma. The number of cases of cancer diagnosed rose from 10 in 1955-

TABLE 4 *Greenland 1955-1975. Surgical Biopsy Specimens Examined during the Period*

	1955-1959	1960-1964	1965-1969	1970-1975	Total
All surgical biopsies, cervical biopsies included	263	2627	4249	6704	14443
Cervical biopsies only	163 (19 %)	330 (20 %)	811 (19 %)	1900 (28 %)	3406 (24 %)
Annual average number of biopsies per 1000 over 15 years	10.2	26.4	33.7	38.4	
Annual average number of cervical biopsies per 1000 women over 15 years	3.9	10.9	14.4	24.2	
Cervical biopsies with invasive carcinoma (percentage of all cervical biopsies)	7 (4.2 %)	11 (3.6 %)	30 (3.7 %)	76 (4.0 %)	132 (3.9 %)
Cervical biopsies with dysplasia and carcinoma in situ (percentage of all cervical biopsies)	4 (2.4 %)	13 (2.4 %)	17 (3.3 %)	193 (10.1 %)	233 (6.9 %)

1959 to 45 in 1970-1974 (Table 2) In 1975 18 new cases were diagnosed the highest number in any year The diagnosis was confirmed histologically in 116 cases, all of them squamous cell carcinoma. At the time of diagnosis one case was clinically so advanced that positive findings at cytology were considered to be sufficient.

The average annual crude incidence rate per 100 000 women over 15 years of age rose from 26.2 for 1955-1959 to 82.6 for 1970-1974 The age-adjusted incidence rate per 100 000 women all age groups, rose from 20.9 for 1955-1959 to 70.3 for 1970-1974

Table 5 shows the observed number of cases of cervical carcinoma in Greenland and the expected number assuming the incidence rate to be the same as in Denmark For the period 1970-1975 there were 63 observed and 22.9 expected cases.

The age-specific rates for cervical cancer in Greenland from 1970 to 1975 are shown in Table 6 The highest incidence rate was seen in the age group 35-49 years with another peak occurring at the age of 60-64 years. The incidence rate for all age groups over 15 years was 95.2 per 100 000 women, and the age-adjusted incidence rate for all age groups was 84.8 per 100 000 At the time of diagnosis, the youngest patient was 17 years old

Table 7 presents the incidence of carcinoma in the individual districts for the period 1968-1975 The expected number of

TABLE 6 *Incidence Carcinoma of the Uterine Cervix Greenland 1970-1975 Annual Age-specific Incidence Rates per 100,000 Indigenous Greenland Women Compared with the Rates from Denmark for 1968-1972 (Provided by the Danish Cancer Registry)*

Age	Greenland 1970-1975	Denmark 1968-1972
15-19	8.5	-
20-24	34.2*	1.5
25-29	64.4*	10.6
30-34	86.4*	27.9
35-39	208.3*	55.3
40-44	183.7*	77.4
45-49	199.2	83.3
50-54	112.0	73.5
55-59	31.4	62.6
60-64	258.7	56.2
65-69	47.7	43.6
70-74	-	46.1
75-79	75.9	36.2
80+	-	41.0
All age groups	53.0	42.5
All age groups over 15	95.2	
All age groups, age-adjusted ("European Standard Population")	84.8	34.3

TABLE 7 *Cervical Carcinoma in Greenland by Districts 1968-1975 Observed and Expected* Number of Cases*

Districts	Number of cases	
	Observed	Expected*
Nanortalik	5	5.4
Jullaneq	5	6.1
Narsaq	4	4.0
Frederikshåb	4	4.3
Godthåb	16	12.8
Sukkertoppen	4	6.0
Holstenborg	6	6.0
Egedsmunde (incl. Kangatsuaq)	8	8.4
Christianshåb	2	2.1
Jakobshavn	6	5.5
Godhavn	1	1.8
Umanak	5	4.5
Lpernavik	4	3.5
Thule	1	1.1
Angmagssalik	5	4.1
Scorebysund	1	0.8
All districts	77	77.0

TABLE 5 *Carcinoma of the Uterine Cervix in Greenland 1955-1975 Observed and Expected* Number of Cases*

Period	Observed	Expected*	O/E ratio
1955-1959	10	13.9	0.7
1960-1964	19	16.7	1.1
1965-1969	25	18.2	1.4
1970-1975	63	22.9	2.7***
Total	117	71.7	1.6***

* Assuming that the population in Greenland had the mortality observed in Denmark

* If crude incidence rate for all Greenland 1968-1975 is applied.

TABLE 8. *Cervical Carcinoma, Dysplasia and Carcinoma in Situ Greenland 1970-1975 Number of Cases in which Cytological Examination was Performed prior to Histological Diagnosis*

	Cytology positive	Cytology negative or non diagnostic	No cytology	Total
Carcinoma	3	5	55	63
Dysplasia + carcinoma in situ	13	3	65	91

cases in each district based on the crude incidence rate of the period for the country as a whole, agreed well with the number of cases actually observed. When comparing the individual districts on the basis of the census of 31st December 1970 a fairly equal age distribution of the female population was found.

Table 8 shows the number of cases in the period 1970-1975 in which cytological examination was performed prior to the histological examination which confirmed the diagnosis. Eight cases of carcinoma (13 per cent) had first had a cytological examination but in only 3 of these cases (5 per cent) did this examination give rise to suspicion.

TABLE 9. *Severe Dysplasia and Carcinoma in Situ of the Uterine Cervix Greenland 1970-1975 Number of Cases Diagnosed in Indigenous Greenland Women A and Age-specific Rates per 100,000*

Age	Cases	Rates
15-19	1	8.5
20-24	4	45.6
25-29	13	193.0
30-34	12	172.7
35-39	10	160.4
40-44	14	233.8
45-49	3	66.4
50-54	1	28.0
55-59	2	61.0
60-64	-	-
65-69	1	48.8
70+	-	-
All over 15	63	93.7

Thirty deaths with a diagnosis of cervical cancer were recorded by reviewing the death certificates from 1967 to 1974.

Dysplasia and carcinoma in situ. In the period 1955-1969 a total of 15 years, 28 cases of dysplasia or carcinoma in situ were diagnosed, whereas a heavy increase was found over the most recent six years, 1970-1975 with a total of 91 new cases (Table 8). All cases were confirmed histologically. The crude incidence rate per 100,000 women over 15 years of age exhibited a rise from 10.5 in 1955-1959 to 137.5 in 1970-1975.

Cases of severe dysplasia and carcinoma in situ observed in the period 1970-1975 as well as age-specific rates, are presented in Table 9. The incidence rose rapidly with age, reaching a plateau at 25-29 years, whereas the incidence rates for the age groups over 45 years were low. The incidence per 100,000 women over 15 years of age was 93.7. The youngest patient was 16 years of age the oldest, 66 years.

A primary cytological examination was performed in 26 cases of dysplasia or carcinoma in situ (29 per cent) during the period 1970-1975 (Table 8); half of the examinations (13) being carried out in 1975.

DISCUSSION

The apparently heavy increase in age-adjusted incidence rates for cervical carcinoma in Greenland (Tables 2 and 6) makes it necessary to consider whether there is a true increase in fresh cases each year or whether the registration has become complete because of better detection methods, possibly combined with more advanced diagnostics.

As will be seen from Table 4 the annual number of cervical biopsies over the years 1955-1959 was very low. It cannot be excluded that cervical cancer has been underdiagnosed during that period, especially in the years prior to 1957 before the surgical ward was established at "Dronning Ingrid's Hospital". For 1960-1964 the average annual number of cervical biopsies per 1000

1959 to 45 in 1970-1974 (Table 2). In 1975 18 new cases were diagnosed the highest number in any year. The diagnosis was confirmed histologically in 116 cases, all of them squamous cell carcinoma. At the time of diagnosis one case was clinically so advanced that positive findings at cytology were considered to be sufficient.

The average annual crude incidence rate per 100 000 women over 15 years of age rose from 26.2 for 1955-1959 to 82.6 for 1970-1974. The age-adjusted incidence rate per 100 000 women, all age groups rose from 20.9 for 1955-1959 to 70.3 for 1970-1974.

Table 5 shows the observed number of cases of cervical carcinoma in Greenland and the expected number assuming the incidence rate to be the same as in Denmark. For the period 1970-1975 there were 63 observed and 22.9 expected cases.

The age specific rates for cervical cancer in Greenland from 1970 to 1975 are shown in Table 6. The highest incidence rate was seen in the age group 35-49 years, with another peak occurring at the age of 60-64 years. The incidence rate for all age groups over 15 years was 95.2 per 100 000 women and the age adjusted incidence rate for all age groups was 84.8 per 100 000. At the time of diagnosis, the youngest patient was 17 years old.

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Christianshåb	2	2.1
Jakobshavn	6	5.5
Godhavn	1	1.8
Umanak	5	4.3
Upernavik	4	3.3
Thule	1	1.1
Angmagssalik	5	4.1
Scoresbyund	1	0.8
All districts	7	77.0

* If crude incidence rate for all Greenland 1968-1975 is applied.

have made it necessary for a great number of women to work outside the home, mainly in the fishing industry service trade and administration. The groups of adolescents in the towns have become greater more unpredictable and more mobile, because of better travelling facilities. At the same time the sexual pattern has changed. Before 1945 young unmarried Greenlanders seem to have been actually rather reticent (Olson 1975 1974) whereas in Greenland today there is extensive sexual practice with early onset of coitus, multiple sexual mates and, since 1968 an exponential increase in the number of venereal diseases (Olson 1974 1976). In 1975 alone 13 new cases of acquired syphilis per 1000 inhabitants were notified (Olson 1976). The frequency of HSV 2 has not been studied, but HLA B12 does not seem to be frequent in the Greenlandic population (Kusmeyer-Nielsen et al. 1971). The incidence of cancer of the penis is low 0.5 per 100,000 males (Nielsen et al.) as against 1.5 per 100,000 in Denmark (Clemmesen 1974).

The fact that the change in epidemiological factors was not followed by an increasing incidence of cervical carcinoma until 1970, is compatible with the prolonged development from carcinoma in situ to invasive carcinoma, at most investigations started to be from 8-14 years (Boyer 1969 Christophersen et al. 1970, Dickerson et al. 1972).

The age-adjusted incidence rate for cervical cancer in Greenland for 1970-1975 (Table 6) is one of the highest in the world (Basthorns et al. 1976) equal to the figures from Cali, Colombia. A well organized survey with a view to detecting cervical cancer and initial stages of the disease has not been instituted in Greenland so far. Although numerous non-organized histological and cytological examinations of the uterine cervix have been carried out in recent years in connection with the above-mentioned gynecological examinations for other purposes (venereal diseases, IUD) the true incidence and prevalence of dysplasia and carcinoma in situ in Greenland have not been clarified. Only 20 per cent of these women had in

1970-1975 had a cytological examination, and, contrary to expectations when better screening procedures were introduced, the percentage of positive biopsies increased (3.3 per cent in 1965-1969 against 10.1 in 1970-1975) (Table 4). More organized casefinding with a view to detecting cervical cancer and its initial stages, seems thus to be well motivated.

Although the Danish Cancer Registry has hitherto not deemed the statistics concerning Greenlandic cases to be sufficiently substantiated for publication, it must be pointed out that correct notification of all malignant cases both in Denmark and in Greenland to the Cancer Registry is of the utmost importance for assessing the incidence of malignant diseases. Only 70 out of the 98 cases of cancer included in the present study as diagnosed between 1937 and 1974 were reported to the Cancer Registry. Out of the 28 non-reported cases, 13 were from the period 1970-1974.

CONCLUSION

A preliminary study on the incidence of carcinoma of the uterine cervix in Greenland revealed rates which were about 2.5 times those found in Denmark, exhibiting an upward trend. It must be presumed that there are still a number of non-diagnosed cases of carcinoma as well as of initial stages of the disease. More organized casefinding is warranted.

This work has been supported by Sygekassernes Hælsesond.

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women over 15 years of age was 10.9 increasing to 14.4 in 1965–1969. These figures do not include a great number of women who underwent diagnostic or therapeutic abrasion over the same period without simultaneous cervical biopsy being performed. Since 1960 many Greenlandic women with gynaecological symptoms have consulted a doctor and cervical biopsies have been carried out to a great extent for diagnostic purposes. It is highly probable that all cases of cervical carcinoma with clinical symptoms have been diagnosed during that period.

From 1965–1969 to 1970–1975 the annual number of cervical biopsies increased by 68 per cent (Table 4). This reflects the great number of gynaecological examinations carried out during recent years in connection with the detection and treatment of venereal diseases and with the insertion of intrauterine contraceptive devices (IUD). During 1971 alone 3 601 cases of gonorrhoea were notified in Greenland as against 1 255 cases in 1965 (*Chief Medical Officer in Greenland* 1968, 1971) and 6 017 IUDs were inserted over the period 1968–1972 (*Ministry for Greenland* 1977). Presumably some asymptomatic cases of cervical cancer and several cases of dysplasia and carcinoma *in situ* must thereby have been detected, and the possibility cannot be excluded that the apparently increased incidence of dysplasia and carcinoma *in situ* in the period 1970–1975 may result mainly from improved histological and cytological registration (Table 8). Cervical/vaginal cytology was hardly ever applied before 1970.

On the other hand a better detection of early asymptomatic carcinomas cannot reasonably explain the doubling of the cancer incidence rates for 1970–1975 as compared with the preceding 10 years (Tables 2 and 6). During this period the number of biopsies revealing invasive carcinoma was 4 per cent of all cervical biopsies, and this was unchanged compared with the preceding periods (Table 4). Only 5 per cent of cases of cancer in 1970–1975 were primarily diagnosed by cytological examination (Table 8)

and while the maximum number of cervical biopsies in a single year was taken in 1971 the annual number of diagnosed cancer peaked in 1975.

The incidence rate for cervical cancer in Greenland in 1970–1975 was about 2.5 times higher than the most recently recorded incidence rate in Denmark (Table 6). The difference between the observed and expected number of cases during this period (Table 5) is statistically significant ($P < 0.0005$). A comparison between age-specific rates for cervical cancer in Greenland for 1970–1975 and the latest calculated rates for Denmark, 1968–1972 reveals significantly higher rates for Greenland in the age groups under 50 years and in the age group 60–64 years ($P < 0.05$) (Table 6).

It is well known that the incidence both of cervical carcinoma and of carcinoma *in situ* is related to early onset of coitus and frequent contacts with multiple sexual mates (Rotkin 1967) which may also have a bearing on the previously demonstrated correlation with low socio-economic status, which was more pronounced earlier (Stamler *et al.* 1967). Antibodies against herpes-simplex virus type 2 (HSV 2) also seem to be more frequent in patients with cervical cancer than in controls (Schneeweiß 1974) and a positive relationship between HSV 2 antibodies, HLA B12 antigen and cervical carcinoma has been demonstrated (Koenig *et al.* 1976). Whether this is true causal relationship or possibly as the relationship with syphilis (Røjel 1953) could be explained by the connection with promiscuity of both factors, remains to be clarified. In a few populations with a high incidence of cervical cancer a considerable frequency of cancer of the penis has been recorded but a direct relationship has never been convincingly demonstrated (Clemmensen 1965).

During the last 25–30 years, the occupational restructuring and the concentration of the population in the Greenland community which previously depended mostly on the hunting trade have given rise to radical changes. Urbanization and industrialization

PLASMA MEMBRANE MOTILITY AND PROLIFERATION OF HUMAN GLIOMA CELLS IN AGAROSE AND MONOLAYER CULTURES

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Carlsson, J. Collins, P. & Brunk, Ulf. Plasma membrane motility and proliferation of human glioma cells in agarose and monolayer cultures. *Acta path. microbiol. scand. Sect. A*, 86 45-55, 1978.

Human glioma cells, growing as spherical colonies in agarose gel, or as monolayers on glass or plastic, were studied with time-lapse cinematography and electron microscopy. The cells in the agarose cultured colonies often had ruffling-like membrane structures which were similar in form although smaller in size, than those observed on monolayer cultured cells. The ruffling-like structures were more frequent at the periphery than in the central regions of the colonies which was in parallel to the proliferation pattern. In time-lapse cinematography it was seen that pinocytotic vacuoles were formed from ruffling membranes in the monolayer cultures. In the transmission electron microscope, such vacuoles were also found near the ruffling-like structures in the agarose cultured cells. In dense monolayer cultures, ruffling and associated pinocytosis were so large events transferred from the margin to the upper surface of the cells. This capacity may be an important property for the ability of the malignant cells to attain extreme high cell densities in monolayer cultures and to grow as colonies in agarose cultures. As has been previously shown, the normal counterpart of the gliomas, the glia cells, cannot grow to high densities: they do not ruffle on their upper cell surface and are unable to grow in suspension or agarose culture.

Key words: Cell culture, electron microscopy, proliferation, ruffling membranes, time-lapse cinematography.

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Recd. ed 23 77 Accepted 10 ix.77

Normal diploid cells in culture generally need a solid support for their growth and are said to be anchorage-dependent (Stoker *et al.* 1968). Only a few diploid cell types (for example stimulated lymphocytes) are capable of multiplication in suspension. This is in contrast to the behaviour of many malignant cell lines which can grow without solid support in

agar gel or suspension (e.g. Eagle *et al.* 1970, Giard *et al.* 1973).

The agar gel culture method was first used as a test for virus transformation. Sanders and Burford (1964), Macpherson and Montagnier (1964) and Eagle *et al.* (1970) investigated several cell types, and found that the plating efficiency in soft agar was usually high for virus-transformed cells, while non-neoplastic

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Received 23.1.77 Accepted 10.5.77

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agar gel or suspension (e.g. Eagle *et al.* 1970, Giard *et al.* 1973).

The agar gel culture method was first used as a test for virus transformation. Sanders and Barford (1964), Macpherson and Montagnier (1964) and Eagle *et al.* (1970) investigated several cell types, and found that the plating efficiency in soft agar was usually high for virus-transformed cells, while non-neoplastic

cells did not form colonies. Eagle *et al* (1970) also showed that high plating efficiency in agar was often accompanied by high terminal cell density and low serum requirement in conventional monolayer cultures. The ability to grow as colonies in agar has thus been correlated to malignancy.

We have previously shown that some plasma membrane activities such as ruffling and associated pinocytosis, appear to be intimately related to cellular proliferation in human glioma and glioma cell cultures growing as monolayers on plastic and glass (Brunk *et al* 1976 Schellens *et al* 1976 Collins *et al* 1977). The aim of the present study was to elucidate whether this relation is also valid for malignant glioma cells when cultured as colonies in agarose gel. A finding of ruffling activity during proliferation in gel culture would lend further support to the notion that a relationship exists between such activity and cell proliferation. Ruffling has hitherto been mainly associated with cellular locomotion on solid substrates (Abercrombie *et al* 1970 Harris 1973).

MATERIAL AND METHODS

Cell Line

The establishment of the human glioma cell line studied (118 MG) has previously been described by Wæstermark *et al* (1973). The cells were normally grown in Eagle's minimum essential medium (MEM) supplemented with 10 per cent bovine calf serum and antibiotics (100 IU/ml penicillin, 50 µg/ml streptomycin and 1.25 µg/ml amphotericin B) at 37 °C in a humid atmosphere containing 5 per cent CO₂.

Agarose Cultured Colonies

Cultivation in the semi solid medium was performed as described by Macpherson (1973) except that agarose was employed instead of agar. Five ml of 0.5 per cent agarose medium, containing 10 per cent tryptose phosphate broth in Eagle's MEM, were poured into 5 cm plastic dishes and allowed to solidify at room temperature. Above this layer 1.5 ml of 0.33 per cent agarose medium containing 10⁴ cells was added. The dishes were incubated at 37 °C in a humid atmosphere containing 5 per cent CO₂. A quantity of 0.5 ml fresh medium (Eagle's MEM + 10 per cent calf serum) was routinely

added on the top of the cultures three times a week during the experiments.

All colonies studied in the light and the electron microscope had diameters in the range of 400–600 µm.

Monolayer Culture

The monolayer cultures were prepared following routine subcultivation (Pontin & Macarty 1968) with a split of 1:4. Cells to be studied in the scanning electron microscope were plated in 5 cm Nunclon® plastic dishes containing round cover slips (diameter 12 mm).

Time Lapse Cinematography

Time-lapse cinematography was carried out on cultures prepared as described above for both agarose and monolayer cultures. The cultures were set up in Sykes-Moore chambers (Sykes & Moore 1959). To the medium was added 25 mM HEPES buffer instead of the sodium bicarbonate-carbon dioxide buffer system. The cultures were filmed at 37 °C at a rate of one frame per 20 sec, using a Leica Special Super 8 camera controlled by a Leitz ST10M control unit and a NR10M mains and recording unit. The film used was Kodachrome 40 type A. The camera was positioned over a Leitz Diavert microscope and the cultures were filmed with a phase contrast × 20 or × 32 objective.

Growth Curves Agarose Cultured Colonies

The growth of the colonies in agarose was determined as described previously by Carlson & Brunk (1977). Individual colonies were measured by means of an ocular grid manipulated by a micrometer screw in the eyepiece of an inverted microscope. The volume of each colony was calculated from the relation $V = 4/3 \pi (ab)^{2/3}$ where a and b were radii measured at right angles. Identification of the individual colonies was made possible by a coordinate system on the bottom of the dishes.

Growth Curves Monolayers

The cell density in the monolayer cultures was measured by cell counts on parallel cultures, using an electronic cell counter (Coulter).

Administration of ³H TdR and Autoradiography of Agarose Cultured Colonies

The cells were labelled with ³H-methyl thymidine (³H TdR) with the specific activity 5 Ci/mM (Radiochemical Centre, Amersham, U.K.).

A quantity of 0.5 ml medium (Eagle's MEM with 10 per cent bovine calf serum) containing 0.5 Ci ³H TdR was evenly spread on the top of the agarose cultures. The dishes were then in

cultured for 24 hours, washed in phosphate buffer solution, fixed in methanol-acetic acid (3:1) for two hours, and kept in 70 per cent alcohol (with the cells remaining in agarose). Areas of about 0.5×1.0 cm were cut with a knife around cell colonies, to permit the agarose piece to be transferred to dehydration bath (10 per cent alcohol) for about two hours. Infiltration in water-soluble plastic (glycol-methacrylate) and moulding were performed as described in Sorra's manual (1971).

Sections 2 μ m thick, were cut with a glass knife, stained with haematoxylin (10 minutes) and finally washed in running tap water.

The sections were processed for autoradiography by the dipping technique (using the Ilford K5 emulsion) and left in the dark for 30 days before development (in Kodak D19-B) and fixation. The number of both labelled and unlabelled cells were counted, using a square mounted in the eyepiece of a Leitz Orthoplan microscope. Using the $100\times$ objective, the square corresponded to $40 \times 80 \mu$ m and was placed in different regions of colonies.

Administration of ^3H TdR and Autoradiography of Monolayers

Monolayer cultures were labelled with ^3H TdR (see above) diluted to a final concentration of $0.01 \mu\text{Ci/ml}$ in medium containing 10 per cent calf serum.

After incubation for 24 hours the cultures were washed four times in phosphate-buffer solution and fixed in methanol-acetic acid (3:1) for 2×30 minutes. The bottoms of the dishes were punched out, coated with Kodak AR 10 stripping film, left in the dark for 14 days at 4°C , developed for 5 minutes in Kodak D-19B, fixed for 10 minutes in Kodak Acid Fixer, washed for 10 minutes in running tap water and dried.

In dense cultures of glioma cells, cell-overlapping was very common. All nuclei could, however, easily be recognized after hydrolysis as described by Carlson *et al.* (1976). This technique provides pronounced focal contrast between the nuclei and the cytoplasm. The cells were hydrolysed in $2 \text{ ml } 0.1 \text{ N HCl}$ at 60°C for 15 minutes, washed in distilled water, stained with haematoxylin for 10 minutes, and then washed in cold running tap water for 15 minutes. The labelling index was determined from measurements on more than 200 cells from each of duplicate dishes.

Transmission Electron Microscopy of Agarose Cultured Glioma Colonies

All colonies studied were fixed 4-5 days after plating (in the phase of exponential growth). Colonies in small pieces of agarose (approximately $3 \text{ mm} \times 3 \text{ mm}$) were fixed for 6 hours in 2 per cent glutaraldehyde in $0.1 \text{ M Na-cacodylate HCl}$ buffer with 0.1 M sucrose (pH 7.2, total osmolality

510 mOsm , ionic osmolality 300 mOsm) at $0-4^\circ\text{C}$ (Brink & Ericsson 1972). They were then post-fixed in 2 per cent osmium in γ -collidine buffer (pH 7.2) for 90 minutes, dehydrated in a graded series of ethanol, stained *en bloc* with arylal acetate in 100 per cent ethanol for 10 minutes and embedded in Epon 812. Thin sections were stained with lead citrate and examined in Jeol 100 C electron microscope operated at 60 kV.

Transmission Electron Microscopy of Glioma Monolayers

The cell cultures were fixed *in situ* in the plastic dishes at 37°C for 60 minutes in the above described aldehyde fixative. This was followed by post-fixation in 1 per cent OsO₄ in $0.15 \text{ M Na-cacodylate HCl}$ buffer and further preparation and embedding as has been previously described (Brink *et al.* 1971). The polymerized blocks were then cut and examined in a Philips 201 electron microscope operated at 60 kV.

Scanning Electron Microscopy of Glioma Monolayers

Cells were fixed for SEM when the cultures were sparse and when they had grown dense and crowded. The cell-bearing coverslips were rapidly transferred from the culture medium to the fixative solution and fixed in the same glutaraldehyde based fixative as was used for the TEM preparation. The fixation was initially carried out for 15 minutes at 37°C during gentle agitation and subsequently continued in a refrigerator at $+4^\circ\text{C}$ for another 1-6 hours. The cells were postfixed in 1 per cent OsO₄ in 0.15 M cacodylate buffer pH 7.2, at room temperature for 90 minutes, dehydrated in a graded series of acetone and critical point dried from CO₂ in a Polaron E 3000 apparatus. Care was taken to avoid drying during dehydration by keeping the specimens well below the surface during all exchanges of dehydration agents and transfer to the critical point drying apparatus. The specimens were mounted on stubs with adhesive tape and coated with a 20 nm thick layer of gold in a Polaron sputter operated at 20 mA and 1.2 kV. After coating, the edges of the coverslips were sealed to the stubs with silver conductive paint, and the specimens were studied in a Jeol JSM 81 microscope at 10 kV or in Jeol 100-C microscope equipped with a scanning attachment at 40 kV. Micrographs were taken with the specimens tilted 45° .

RESULTS

Growth of Agarose Cultures

The growth of the 118-MG glioma colonies was nearly exponential up to about 10 days

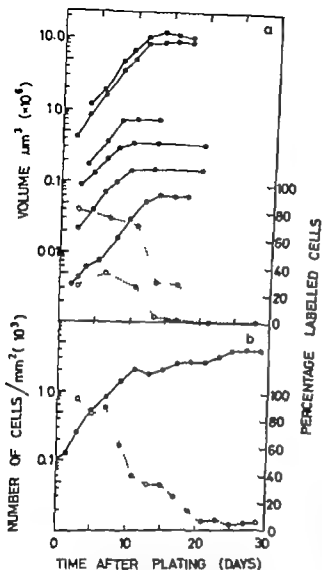


Fig 1 Labelling indices (dotted lines) after 24 hours incubation with ^3H TdR together with growth curves (solid lines) for a) glioma colonies (118 MG) cultured in agarose and b) glioma cells (118 MG) in monolayer culture. The upper dotted line in Fig a refers to labelling indices measured in the periphery of the colonies, while the lower curve refers to measurements in areas situated 150–200 μm from the outer surface of the colonies.

after plating. In parallel to this, the labelling index in both peripheral and central regions of the colonies decreased dramatically after ten days (Fig 1 a). The population doubling time in the exponential phase was about 50 hours. All colonies studied below in the transmission electron microscope and with time lapse cinematography were in this phase.

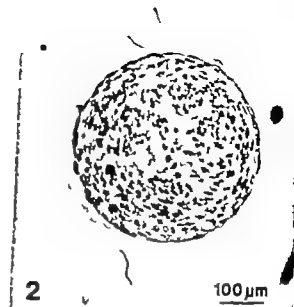


Fig 2 A 2 μm thick section of a plastic embedded glioma colony (fixed 4 days after plating) stained with haematoxylin and eosin.

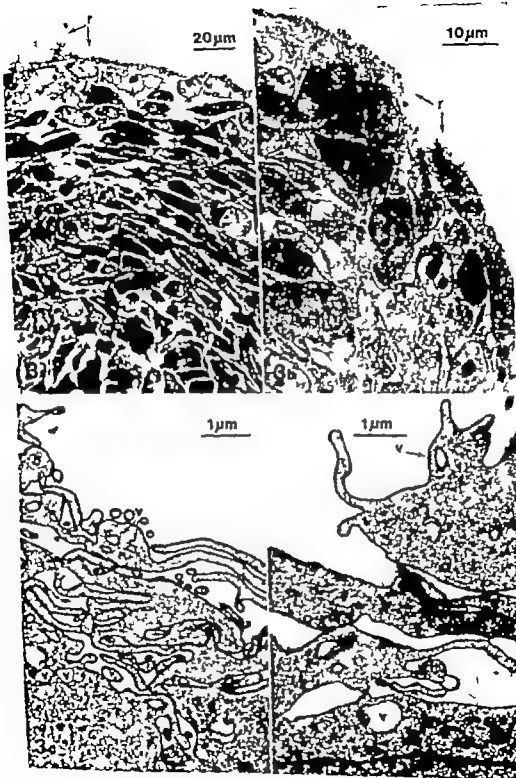
Growth of Monolayer Cultures

The growth curve for the glioma cells 118-MG in monolayer culture (Fig 1 b) showed the same characteristics as the growth curves for several other lines of glioma cells which have previously been reported from our laboratories (Westermarck 1973). The initial, nearly exponential, phase with high labelling indices, was followed by a plateau phase, with low labelling indices, at a density corresponding to about three times that of confluence. The population doubling time was 30 to 40 hours during the exponential growth phase.

Transmission Electron Microscopy of Agarose Cultured Colonies

Although the glioma colonies were nearly spherical (Fig 2) the shape of the individual

Fig 3 Transmission electron microscope pictures of glioma colonies cultured in agarose. Note the cellular organisation with ruffle and vacuole rich cells at the periphery. Fig a and b show large rufflings (marked with r) on these cells. Fig c and d show details of large rufflings. Cytoplasmic vacuoles are seen together with acule-like formations associated with the ruffles (marked with * in Fig. c and d).



4. Arterial mural cell, Sect. A, Fig. 1

cells was very irregular (Fig 3 a and b). The cells often had laminar ruffle-like structures (Fig 3 c and d). Vacuoles could frequently be found in direct relation to these structures. The ruffle-structures were most frequent on the peripheral cells although similar but smaller structures could be found on cells inside the colonies at distances up to 200 μm from the surface (c.f. Fig 4).

Scanning and Transmission Electron Microscopy of Monolayer Cultures

Glioma cells growing at low densities (less than 10^3 cells/ mm^2) in monolayer culture usually showed two or more ruffling areas along their periphery as seen in the scanning electron microscope (Fig 5 a). Transmission electron microscope preparations of these cells showed the ruffles as thin cytoplasmic lamellae (Fig 6 a) reminiscent of the lamellar structures seen on peripheral cells in the agarose cultured colonies (Fig 3 c and d). As seen in Fig 6 vacuoles were found at the base of the ruffles.

In dense monolayer cultures (with about $2 \cdot 10^3$ cells/ mm^2) the cells continued their ruffling activity despite cell-cell contacts, and now ruffles appeared on the upper cell surface (central ruffles) (Fig 5 b and c and Fig 6 b). In the scanning electron microscope it was seen that the number of blebs, microvilli and invaginations on the cell surfaces were high on some cells and low on others and that the cultures gave a heterogeneous impression.

Time-lapse Cinematography of Agarose Cultured Colonies

Single cells moulded in agarose gel were rounded and often showed a protuberance at one point where microspike and ruffle-like membrane activity could be seen. Thus the suspended single cells appeared to specialize an area for similar activities as in sparse monolayer cultures, where rufflings are associated with the leading lamellae (e.g. Harris 1973). In larger colonies containing several cells the membrane of the peripheral cells was still very changeable and ruffling like



Fig 4 Transmission electron microscope picture of a ruffle-like structure inside a glioma colony. The distance from the colony surface was about 50 μm .

structures as well as blebs were frequently seen. The ruffle-like structures were smaller on agarose cultured cells than on monolayer cultured cells.

It was not possible to follow pinocytosis in the agarose cultures by time lapse cinematography owing to light scattering and interference patterns at the periphery of the colonies. Cell divisions were however seen in the periphery of the colonies and were accompanied by active blebbing as in monolayer cultures. The blebs could persist for up to an hour before decreasing and ceasing. Blebs sometimes also occurred when no cell division could be detected.

Time-lapse Cinematography of Monolayer Cultures

In cultures with low densities the glioma cells usually showed two or more ruffles along their leading edge during migration. The ruffles were either broad and extensive or small and tuft like. In dense cultures, tuft like

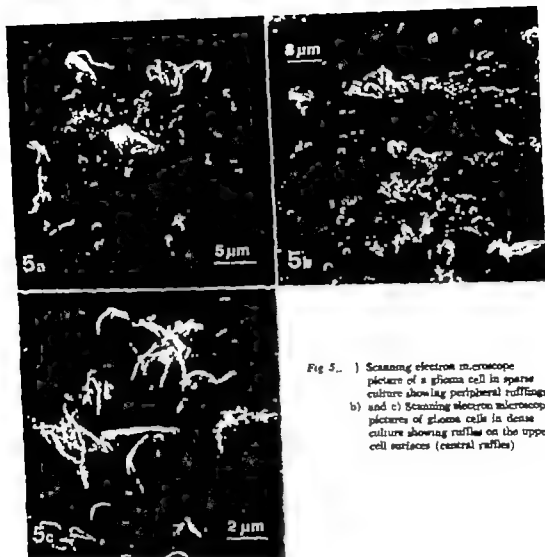


Fig 5. a) Scanning electron microscope picture of a glioma cell in sparse culture showing peripheral ruffings. b) and c) Scanning electron microscope pictures of glioma cells in dense culture showing ruffles on the upper cell surfaces (central ruffles)

ruffles were seen on the upper surface of the cells (central ruffles). These observations were wholly in parallel with the observations in the scanning electron microscope.

The ruffles gave rise to the formation of pinocytotic vacuoles which moved towards the perinuclear area during absorption within the cytoplasm. This vacuole transport was, in dense cultures, also seen after formation of central ruffles.

Cell divisions were seen with the formation of blebs in both dense and sparse cultures. The cells did not form a regular monolayer

but stretched over each other as malignant cells usually do *in vitro*. As in the scanning electron microscope the glioma cells gave a heterogeneous impression. Some cells revealed intense membrane activity while others at times seemed rather inactive.

DISCUSSION

Recent findings in monolayer cultures of human glioma and glioma cells have suggested an association between plasma membrane motility in the form of ruffling and macrophago-

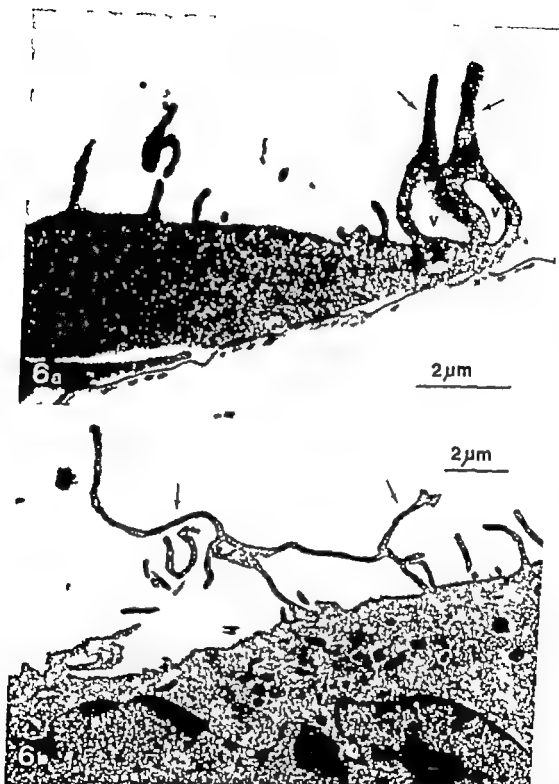


Fig 6 a) Transmission electron micrograph of a glioma cell in sparse monolayer culture showing a section through a leading lamella. Note the large ruffle parts (arrows) and, probably placotytic vacuoles (V) b) A transmission electron micrograph through a ruffle-like structure (arrows) on the upper surface of a glioma cell in a post-confluent culture. Note the presence of transparent vacuoles (V) in the neighbouring area.

cytomes, and entry into or progression through the cell cycle (Brunk *et al* 1976, Schellens *et al* 1976 Collins *et al* 1977). The finding that similar membrane structures exist in fast growing agarose cultured colonies of glioma cells further strengthens this relationship. It also shows that ruffling is not confined to cells moving on a solid substrate. The ruffling structures were most common in the periphery of the colonies, which is in parallel with proliferative pattern. It has previously been demonstrated that proliferation decreases with the distance from the surface of the glioma spheroids, as did also the volumetric fraction of electron transparent cytoplasmic vacuoles - a group which probably consists to a high per cent of pinocytotic vacuoles (Carlson and Brunk 1977) (see Fig. 7).

The ability of the malignant glioma cells to continue ruffling and pinocytosis in confluent monolayer cultures is of special interest since the normal glia cells, which exhibit density dependent regulation of both growth and movement (Porter *et al* 1969 Collins *et al* 1977) do not have this ability. Thus glia in confluent cultures may lack a method for internalization of nutrients, which may in turn be the reason for the inhibition of proliferation under such conditions. In fact, Holley (1974) suggested that density-dependent regulation of growth is simply limited by limitations in the nutrition supply.

The appearance of ruffles on the upper cell surface (central ruffling) on malignant cells has previously been reported for both "spontaneously" transformed (e.g. Gonda *et al* 1976) and virus-transformed cells (e.g. Ambrose *et al* 1975) and has also been associated with pinocytosis and density-independent growth (Lisely 1972).

Several scanning electron microscopical studies have been devoted to the surface structure of mammalian cells in culture. The proliferative state of the cells has, however, not always been reported. In two studies on cells which were clearly shown to be proliferative (Porter *et al* 1973 Anthon *et al* 1975) no extensive ruffling activity of the type seen in Figs. 3-6 could be observed. It is

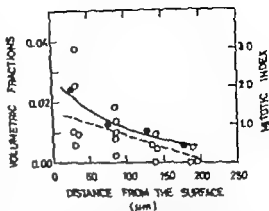


Fig. 7 The volumetric fraction of the cytoplasm which contained vacuoles with electron transparent material (filled circles and dashed line) together with the mitotic index (filled circles and solid line) as a function of depth in the agarose cultured colonies (with permission from Carlson and Brunk, Acta path. microbial. scand. Sect. A, 85 183-192, 1977).

not clear whether this reflects a genome difference in membrane motility between their and our cells, or whether it is to some degree dependent on differences in the preparation technique. It is, for example well known that the preservation of membrane extensions is very sensitive to variations in the fixation procedure and is also easily altered by a temperature decrease or a pH increase of the medium prior to the fixation procedure (Bell *et al* 1975 Arbogh *et al* 1976).

The cellular components directly involved in membrane motility are the microtubular and microfibrillar systems (Goldman *et al* 1973 Allison 1973) but unfortunately little is known about the control of their formation and action. One of the systems which tentatively may be directly or indirectly involved in the control of the cytoskeletal and cytomuscular systems is cAMP and its regulatory enzymes. Many reports have appeared concerning the effect of these substances on both cellular form and growth (see for example, Chapolowski *et al* 1975 and Billingham 1976) but no explanation of the association of ruffling like plasma membrane motility with cell multiplication can be obtained from these data. Since it appears that ruffling is a

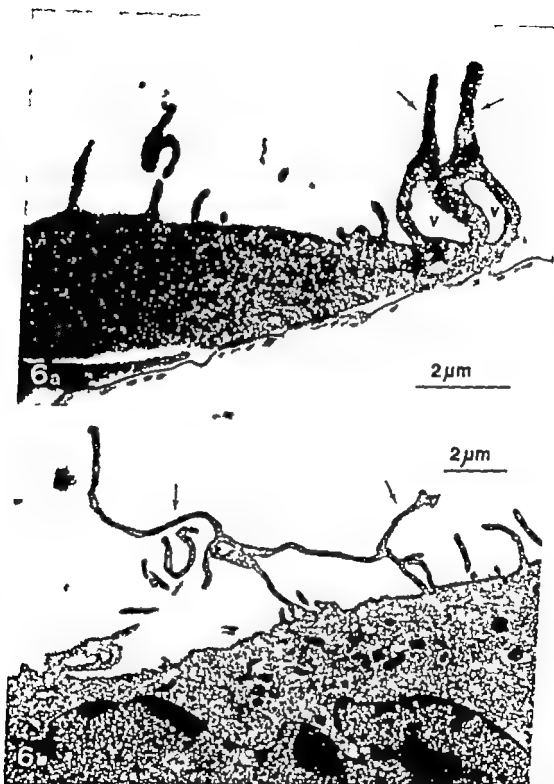


Fig 6 a) Transmission electron micrograph of a gloma cell in sparse monolayer culture showing a section through a leading lamella. Note the large ruffle parts (arrows) and, probably pinocytotic, vacuoles (V) b) A transmission electron micrograph through a ruffle like structure (arrows) on the upper surface of a gloma cell in a post-confluent culture. Note the presence of transparent vacuoles (V) in the neighbouring area.

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prerequisite for the initiation or progress through the cell cycle we foresee that membrane studies on synchronized cells soon after addition of growth stimulating agents will give additional knowledge in the causal analysis.

The work has been financially supported by the Swedish Cancer Society the Swedish Medical Research Council and the Cancer Society of Stockholm

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bone dynamics using tetracycline double-labelling. The bone changes in the hypothyroid patients were compared to normal controls and to sex and age matched hyperthyroid patients in order to increase the understanding of the influence of thyroid hormone(s) on bone.

PATIENTS AND METHODS

The investigation comprised 14 consecutively admitted patients with hypothyroidism: 13 women aged 53-77 years (mean 54) and 1 man aged 68 years. The diagnosis was based upon determination of serum thyroxine (0-38 nmol/l (mean 15) normal range 30-120) serum tri-iodothyronine uptake test (63-92 arb. units (mean 75) normal range 91-109) serum thyroid stimulating hormone (above 20 U/l in all patients, upper normal limit 7 U/l) and absolute ¹³¹I iodine uptake (0.4-3.8 µg/h (mean 1.3) normal range 0.8-3.0). Spontaneous primary hypothyroidism was found in 13 of the patients. Hypothyroidism after subtotal thyroidectomy was found in 1 patient with normal concentrations of calcium, phosphorus and immunoreactive parathyroid hormone. All patients had serum creatinine values within normal range after restoration of a normal metabolic state.

The normal control group for bone morphometry comprised 27 individuals presenting the same age and sex distribution and selected from our series of normal subjects (Ajlert *et al.* 1978). The normal control group for dynamic bone parameters (tetracycline labelling) comprised 11 healthy women aged 19-59 years (mean 27).

The hyperthyroid group comprised 14 untreated patients selected from our series of hyperthyroid patients (Ajlert and Skovsted 1977) to be age and sex matched to the hypothyroid patients.

Double-labelling with tetracycline (Frost 1969) as performed in all patients and volunteers. Bony spurs were obtained from the right iliac crest (Borcher *et al.* 1964). Morphometric analysis was performed on decalcified and undecalcified stained sections. The following parameters were measured (Ajlert *et al.* 1978):

4.1 Cortical Bone

Mean vertical width (MCH) as the mean of ten measurements of external and internal cortex using conventional micrometric eyepiece.

Porosity of external and internal cortical lamella (POR) in per cent of cortical bone area.

Mean perpendicular lacunae (POL) as the mean product of length and width of 50 randomly selected lacunae.

Canals in cortical bone with active osteoclastic

resorption (CAR) in per cent of total number of canals.

B In Trabecular Bone

Absolute volume of trabecular bone (AVTB) in per cent of total bone area.

Osteoid covered surfaces (OS) in per cent of total trabecular bone surfaces.

Osteoid volume (OV) in per cent of AVTB.

Mean width of osteoid surface (WOS) as the mean of four extreme measurements in all surfaces covered with osteoid.

Trabecular osteoclastic resorption surfaces (RS) in per cent of total trabecular bone surfaces.

Linear calcification rate (CR) as the mean distance between the fluorescent tetracycline lines in all double-labelled zones divided by the number of days between tetracycline labelling.

Active trabecular calcification surfaces (ATCS) as tetracycline labelled surfaces in per cent of total trabecular bone surfaces.

Active osteoid calcification surfaces (AOCs) (= calcification fronts) as tetracycline labelled surfaces in per cent of osteoid covered surfaces.

The amount of bone mineralized per day (*bone mineralization rate*) was estimated from the product of CR and ATCS.

The statistical significance of differences in group means was determined by the Wilcoxon test for two samples and correlation coefficients by Spearman rank correlation (R).

RESULTS

The morphometric analysis of cancellous and cortical bone in hypothyroid patients, hyperthyroid patients and normal controls is given in Table 1 a. The statistical significance of differences in group means is shown in Table 1 b.

Amount and structure of bone AVTB was normal in the hypothyroid patients. In the hyperthyroid patients AVTB was slightly decreased ($p < 0.10$) compared both to normal controls and to the hypothyroid patients. In cortical bone MCW was increased both in the hypothyroid and in the hyperthyroid patient group. The cortical porosity (POR) was normal in the hypothyroid patients and markedly increased in the hyperthyroid patients.

Amount and shape of osteoid seams WOS was decreased both in the hypothyroid and in the hyperthyroid patients, whereas no significant changes were found in OS or OV. In the

MORPHOMETRIC AND DYNAMIC STUDIES OF BONE CHANGES IN HYPOTHYROIDISM

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Mosekilde L. & Nielsen, F. Morphometric and dynamic studies of bone changes in hypothyroidism. Acta path. microbiol. scand. Sect. A 86: 56-62, 1978

Bone biopsies were performed after tetracycline double-labelling by transfixing the right iliac crest in fourteen hypothyroid patients. The bone changes in cortical and trabecular bone were determined by simple measurement and point counting on decalcified and undecalcified stained sections and compared to normal controls and to sex and age matched hyperthyroid patients. The amount of trabecular bone and the cortical porosity were unchanged in the hypothyroid patients compared to normal controls, whereas the mean cortical thickness was increased. The amount of osteoid and the length of the osteoid seams were normal whereas the mean width of the osteoid seams was decreased. The linear calcification rate in cancellous bone was decreased as were the active calcification surfaces (tetracycline-labelled) and the percentage of osteoid covered surfaces active in mineralization. The osteoclastic resorption surfaces were unchanged in trabecular bone whereas the osteoclastic activity in cortical bone was decreased. The osteocytic osteolysis was normal. The bone changes in hypothyroidism were opposite to the changes in hyperthyroidism characterized by a very low bone turn-over with a reduced osteoid apposition and bone mineralization rate, an inactive osteoclastic resorption in trabecular bone and a decreased osteoclastic resorption in cortical bone.

Key words: Hypothyroidism, bone turn-over, morphometric and dynamic studies.

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Received 20.v.77 Accepted 21.ix.77

The histomorphometric and dynamic bone changes in hyperthyroidism have previously been described in detail (Meunier *et al.* 1972; Nielsen and Mosekilde 1977; Mosekilde *et al.* 1977). The results demonstrate that an excess of thyroid hormone(s) stimulates bone formation and bone resorption followed by a decrease in the amount of trabecular bone, an increase in cortical porosity and by mobilisation of bone mineral.

The bone changes in hypothyroidism are far less known. Microradiographic studies in hypothyroid dogs (Jousey and Detenbeck

1969) have demonstrated a decrease in bone formation and bone resorption surfaces, which was normalized after thyroxine therapy. Rasmussen and Bordier (1974) described the bone changes in 5 hypothyroid patients by quantitative histological methods and reported a marked decrease in both resorption and formation surfaces and in osteocytic osteolysis followed by a slight increase in the amount of cancellous bone.

The aim of the present study was to describe the bone morphology in hypothyroidism in detail using undecalcified bone sections and the point count principle and to study

TABLE 1b. Statistical Significance of Differences in Group Means (Wilcoxon test for two Samples)

	Hypothyroid/Normal p	Hyperthyroid/Normal p	Hypothyroid/Hyperthyroid p
Trabecular bone			
AVTB	n.s.	< 0.10	< 0.10
OS	n.s.	n.s.	< 0.05
OV	n.s.	n.s.	< 0.10
WOS	< 0.01	< 0.01	n.s.
RS	n.s.	< 0.01	< 0.01
CR	< 0.01	< 0.01	< 0.01
ATCS	< 0.01	n.s.	< 0.01
AOCs	< 0.01	n.s.	< 0.01
Cortical bone			
MCV	< 0.01	< 0.05	n.s.
POB	n.s.	< 0.01	< 0.01
CAR	< 0.05	< 0.01	< 0.01
POL	n.s.	< 0.05	n.s.

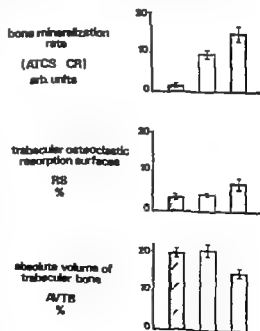
hyperthyroid patients OS was significantly ($p < 0.05$) and OV slightly but insignificantly ($p < 0.10$) decreased compared to the hyperthyroid patients.

Bone resorption activity In cancellous bone RS was normal in the hypothyroid patients and increased in the hyperthyroid patients. In cortical bone POL was normal in the hypothyroid patients and slightly increased in the hyperthyroid patients. CAR was decreased in the hypothyroid patients and markedly increased in the hyperthyroid patients.

Dynamic bone parameters (tetracycline labelling) Both the linear calcification rate (CR) and the extension of the tetracycline labelled surfaces (ATCS) were decreased in the hypothyroid patients. CR was increased in the hyperthyroid patients, whereas ATCS was normal. The percentage of osteoid covered surfaces active in mineralization (AOCs) was decreased in the hypothyroid patients and normal in the hyperthyroid patients.

Bone turn-over Fig 1 gives bone mineralization rate (ATCS \times CR) and bone resorption (RS) in trabecular bone in hypothyroid, normal and hyperthyroid individuals, together with the amount of trabecular bone. The mean bone mineralization rate in the hypothyroid patients was found to be very

low (16% of normal mean, $p < 0.01$) whereas the trabecular osteoclastic resorption surfaces were normal. The mean bone mineralization rate in the hyperthyroid patients



□ hypothyroid □ hyperthyroid □ normal controls

Fig 1 Trabecular bone mass and turn-over in hypo- eu- and hyperthyroid individuals

TABLE 1. *Morphometric and Dynamic Analyses of Trabecular Bone and Cortical Bone in Hypothyroid Patients as Compared with Sex and Age Matched Hyperthyroid Patients and Normal Controls*

Tubercular tons	AVTB %	OS %	OV %	WDS mm	RS %	GR* mm/day	ATCS* %	AOC3* %
Hypothy	19.6	14.0	1.5	6.4	3.7	0.56	4.2	28.7
SE (N)	1.5 (13)	1.8 (13)	0.4 (13)	0.7 (14)	0.5 (13)	0.08 (10)	1.4 (12)	8.9 (12)
Norm.								
Hypothy	20.2	16.7	1.8	8.7	4.0	0.67	14.5	80.3
SE (N)	1.4 (27)	1.4 (27)	0.2 (27)	0.4 (27)	0.5 (27)	0.02 (32)	1.1 (32)	4.7 (31)
Hypothy	15.5	22.7	2.8	6.5	7.3	0.85	18.7	89.4
SE (N)	1.3 (15)	2.9 (14)	0.5 (14)	0.5 (10)	1.1 (14)	0.06 (11)	3.4 (10)	8.1 (10)
Central tons								
Hypothy								
SE (N)								
Norm.								
Hypothy								
SE (N)								
Hypothy								
SE (N)								

Normal controls not sex and age matched

TABLE 1 b. Statistical Significance of Differences in Group Means (Wilcoxon test for two Samples)

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Trabecular bone			
AVTB	n.s.	< 0.10	< 0.10
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OV	n.s.	n.s.	< 0.10
WOS	< 0.01	< 0.01	n.s.
RS	n.s.	< 0.01	< 0.01
CR	< 0.01	< 0.01	< 0.01
ATCS	< 0.01	n.s.	< 0.01
AOCs	< 0.01	n.s.	< 0.01
Cortical bone			
MCW	< 0.01	< 0.05	n.s.
POR	n.s.	< 0.01	< 0.01
CAR	< 0.05	< 0.01	< 0.01
POL	n.s.	< 0.05	n.s.

hyperthyroid patients OS was significantly ($p < 0.05$) and OV slightly but insignificantly ($p < 0.10$) decreased compared to the hyperthyroid patients.

Bone resorption activity In cancellous bone RS was normal in the hypothyroid patients and increased in the hyperthyroid patients. In cortical bone POL was normal in the hypothyroid patients and slightly increased in the hyperthyroid patients. CAR was decreased in the hypothyroid patients and markedly increased in the hyperthyroid patients.

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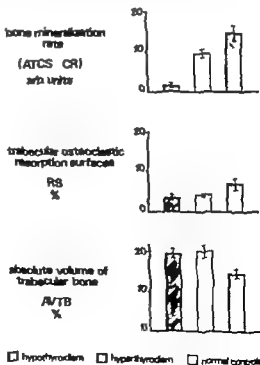


Fig 1 Trabecular bone mass and turn-over in hypo- eu- and hyperthyroid individuals

was found to be increased (152 % of normal mean, $p < 0.02$) as were the trabecular osteoclastic resorption surfaces.

The prevalence of abnormal morphometric bone parameters in the hypothyroid patients is given in Table 2 within 95 % confidence limits. It is seen that a decrease in the linear calcification rate (CR) and a decrease in the percentage of osteoid covered surfaces active in mineralization (AOCS) are the most prominent features in the hypothyroid bone disease. The prevalence of abnormal bone parameters in hyperthyroid patients has previously been reported (Melsen and Mosekilde 1977).

TABLE 2. Prevalence of Abnormal Morphometric Bone Parameters (Exceeding 2 SD from Normal Means) in Hypothyroid Patients.

	%	95 % confidence limits
<i>Trabecular bone</i>		
Decreased WOS	21.4	4.6-50.8
Decreased CR	60.0	26.2-87.8
Decreased ATCS	33.3	9.9-65.1
Decreased AOCS	75.0	42.8-94.5
<i>Cortical bone</i>		
Increased MCW	37.5	17.8-64.9
Decreased GAR	0.0	

Interrelationships between morphometric bone parameters in the hypothyroid patients
Correlations between OS and OV ($R = 0.72$, $p < 0.02$) and between ATCS and AOCS ($R = 0.89$, $p < 0.001$) were found significant. No other significant correlations were found.

DISCUSSION

The present study demonstrates that the amount of bone mineralized per day (bone mineralization rate) estimated as the product of CR and ATCS is decreased in hypothyroidism and increased in hyperthyroidism. The mean age in the control group for dynamic parameters (CR, ATCS, AOCS) was

in the present study considerably lower than in the patient groups. This might influence the results. It was, however, impossible to demonstrate any significant correlation between age and CR ($R = 0.01$), ATCS ($R = 0.04$) or AOCS ($R = -0.21$) in the 32 normal controls aged 19-56 years.

The significant increase in the mean extension of the osteoid covered surfaces (OS) and the slight increase in the mean volume of osteoid (OV) in the hyperthyroid patients compared to the hypothyroid patients suggests that the influence of thyroid hormone(s) on the formation rate of osteoid is or has been slightly more pronounced than the influence on bone mineralization rate. The difference between osteoid formation rate and bone mineralization rate is small, however, both in the hypothyroid and in the hyperthyroid patients. This is demonstrated by the fact that the deviation from normal in OV is insignificant in both groups.

The mean width of the osteoid seams (WOS) was decreased in both patient groups. In the hypothyroid patients this might be explained by an initially more pronounced decrease in the linear apposition rate of osteoid than in the linear calcification rate (CR) caused by the lack of thyroid hormone(s). In the hyperthyroid patients the linear calcification rate may, on the other hand, initially have been slightly more increased than the linear apposition rate of osteoid because of the increase in the calcium phosphorus product found in hyperthyroidism (Mosekilde and Christensen 1977).

The decrease in AOCS in the hypothyroid patients and the normal AOCS in the hyperthyroid patients demonstrates that the osteoid seams are rather inactive in the absence of thyroid hormone(s) whereas the mineralization activity of the osteoid is normal in conditions with an excess of thyroid hormone(s). The observed decrease in AOCS in the hypothyroid patients was caused by a decrease in ATCS and not by an increase in OS. The close relationship between changes in ATCS and changes in AOCS was further supported by the demonstrated positive correlation be-

tween AOCS and ATCS in the hypothyroid patients.

The trabecular osteoclastic resorption surfaces (RS) were found to be normal in hypothyroidism. The resorption surfaces were identified as scalloped interruptions of the lamellar structure, with or without osteoclasts. Thus, the parameter expresses the active as well as the inactive bone resorption. We do not distinguish between active and inactive resorption surfaces because the osteoclasts are very mobile and move across the bone surface (Moser *et al.* 1976). The measured length of the erosion perimeter on trabecular bone constitutes the static parameter of bone resorption. The amount of bone removed in a unit of time depends, however, not only on the extension of the resorption surfaces but also on the activity of the single osteoclasts. Furthermore, it is most likely that the inactive Howship's lacunae persist on the trabecular surfaces for a longer time in low turn-over states because of a delay in the subsequent formation of new bone. The bone resorption rate cannot be measured directly as the bone mineralization rate but has to be estimated from changes in bone mass and bone mineralization rate. The markedly reduced bone mineralization rate and the normal amount of trabecular bone in hypothyroidism indicates that the osteoclastic resorption is rather inactive.

The trabecular osteoclastic resorption surfaces (RS) in the hyperthyroid patients were increased. The slight ($p < 0.10$) decrease in the mean volume of trabecular bone in spite of an increased bone mineralization rate demonstrates that the osteoclastic resorption is very active.

The osteoclastic resorption in cortical bone (CAR) was decreased in the hypothyroid patients and increased in the hyperthyroid patients. This is in agreement with the finding that the osteoclasts in cortical bone are highly sensitive to thyroid hormone(s) (Melsen and Mosekilde 1977) and that an increase in CAR is the main reason for bone mineral mobilization in hyperthyroidism (Mosekilde *et al.* 1977).

The mean size of the periosteocytic lacunae (POL) was normal in the hypothyroid patients and slightly increased in the hyperthyroid patients. Rasmussen and Bordier (1974) have reported a decrease in the osteocytic osteolysis in hypothyroidism. They have, however, expressed the osteocytic osteolysis as the relative number of osteocytic lacunae with ill-defined borders and irregular enlargement in undecalcified bone sections.

The mean cortical width (MCW) was increased in both patient groups compared to normal controls. This might be explained as an artefact due to a higher incidence of oblique orientated bone sections in the biopsy material compared to the control material which was mainly removed at autopsy (Melsen *et al.* to be published).

The present investigation characterizes the bone changes in hypothyroidism as a low turn-over state with a reduced osteoid apposition and bone mineralization rate, an inactive osteoclastic resorption in trabecular bone and a decreased osteoclastic resorption in cortical bone. The changes are in many aspects opposite to those found in hyperthyroidism.

The demonstrated decrease in bone turn-over in hypothyroidism may explain the reduced responsiveness of bone to parathyroid hormone (Jowsey and Detenbeck 1969; Castro *et al.* 1975) to calcitonin (Jowsey and Detenbeck 1969) and to 1,25-dihydroxycholecalciferol (Parfitt *et al.* 1977) in the hypothyroid state. Furthermore, an impaired ability to tolerate hypercalcaemic or hypocalcaemic stress have been reported in thyroxine depleted animals (Jowsey and Detenbeck 1969). Thyroid hormone(s) appears, therefore, to be very important in maintaining calcium homeostasis by regulating the level of bone turn-over.

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AN EVALUATION OF THE QUANTITATIVE PARAMETERS APPLIED IN BONE HISTOLOGY

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F. Melsen, B. Melsen and L. Mosekilde. An evaluation of the quantitative parameters applied in bone histology. *Acta path. microbiol. scand. Sect. A*, 86 63-69 1978.

An evaluation of the following histomorphometric parameters about the trabecular bone volume (ABV) osteoid surfaces (OS) osteoid volume (OV) resorption surfaces (RS) and mean size of periosteocytic lacunae (POL) was carried out. Double determination on sections, obtained from an area 2 cm below the summit and 2 cm behind the anterior superior spine of the iliac crest from 20 normal individuals aged 20-40 years, showed that the precision of method by far outweighed the inter-individual variation. Parameters obtained from 9 different localizations within the iliac bone revealed that the volume of bone was the only parameter which was influenced if the bone specimen was removed more than 2 cm below or behind the standard localization. Randomly selected sections alternately stained with Masson trichrome and toluidin blue were used for the study of the influence of the staining method on the osteoid parameters. It was shown that Masson trichrome staining would generally result in a slightly higher value of osteoid surfaces. There was, however, high correlation between the parameters obtained with the two methods. (OS $r = 0.96$ OV $= 0.87$)

Key words: Iliac bone histomorphometry errors.

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Received 20 77 Accepted 12 77

In the evaluation of a quantitative analysis of bone biopsies it is of the utmost importance to be able to disclose with great certainty even minor deviations. The significance with which a diagnosis of metabolic bone diseases can be carried out is to a large extent dependent on the validity of the normal reference material with which the bone biopsy has to be compared. (Mewster 1967) The inter as well as the intra-individual variations are, however, also of the greatest importance. Whereas the inter-individual variation is of importance in the primary diagnosis, the evaluation of changes in the bone status in the individual

patient during the development of a disease or during treatment is completely dependent on the intra-individual variation.

It is well known that the amount of trabecular bone is to a great extent dependent on the localization of the bone to be examined (Courpron 1972, Mewster *et al* 1973). For this reason, bone biopsies which are to be compared have always been removed from standard localizations.

As, for practical reasons, it is not possible to remove consecutive biopsies from exactly the same localization in the same individual, it is important to study variations in the various parameters obtained from biopsies

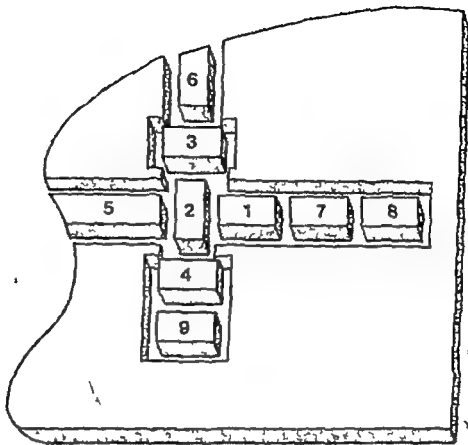


Fig 1 Tissue block from the iliac bone divided into minor bone blocks.

from adjacent areas. Thus, as well as an analysis of the influence on the volume of the orientation of the section is described in the present study. The error included in the method will be calculated on the basis of double determination on the same section and the influence of different staining methods are evaluated on adjacent sections.

MATERIAL AND METHODS

The material for this study comprised tissue blocks from the right and left iliac crests removed at autopsy from 20 individuals between 20 and 40 years of age, who were included in the normal material described elsewhere (Melsen *et al* 1978). Further iliac crest biopsies from 20 patients suffer-

ing from hyperthyroidism were included in the study.

The following parameters were measured: the amount of trabecular bone (AVTB), the extent, amount and width (OS, OV, WOS) of osteoid, osteoclastic resorption surfaces (RS) and the mean size of periosteocytic lacunae (POL) (Melsen *et al* 1978). With the purpose of studying the error included in the histomorphometry, double determinations of sections from the above-mentioned normal individuals were performed at intervals of 3-4 months.

In order to study the influence of the localization of the biopsy and of the orientation of the sections on the results of the quantitative analysis, 10 tissue blocks removed at autopsy were divided into nine minor trans-cortical bone blocks (1 cm wide and 0.5 cm high). Fig 1 illustrates the areas to be examined.

One block (No 2, Fig. 1) comprised exactly the

area where normal bone biopsies are obtained, i.e. 2 cm below and 2 cm behind the antero-superior iliac spine. This block was serially sectioned in vertical plane perpendicular to the bone surface.

To study the effect of variation in the orientation of the section, three bone blocks (Nos. 1, 3 and 4, Fig. 1) were removed adjacent to the biopsy area and superior, one inferior and one posterior to the first block removed. From all these blocks serial sections were cut horizontally.

Additional bone blocks were cut out further posteriorly (Nos. 7, 8, Fig. 1) so the normal biopsy location as well as anteriorly (No. 5, Fig. 1) superiorly (No. 6, Fig. 1) and inferiorly (No. 9, Fig. 1) with the purpose of studying the influence of the localization on the result.

In ten cases, additional tissue blocks were removed from the standard localization to study the variations between right and left side.

Finally the influence of the staining methods on the determination of the osteoid parameters (osteoid surface and osteoid volume) was studied on randomly selected sections from the patients suffering from hyperthyroidism where these values are of importance. The sections were alternately stained with Mason trichrome and Toluidin blue, and the results of the quantifications were compared.

Statistics

Error of the mean value. The method error

$$s(\bar{x}) = \sqrt{\frac{\sum (x_1 - \bar{x})^2}{2N}}$$

represents the uncertainty of

the individual observation of a variable and was determined from the duplicate measurements. The statistical description of the distribution of the differences (d) between duplicate measurements was then carried out, and the means (d) compared to zero in order to disclose systematic errors (Table 1). The variation observed by double determination (variance comp. No. I) was further compared to the inter-individual variation (variance comp. II) by means of a two-way analysis of variance (anova I) (Sokal and Rohlf 1969).

Intra-individual variation (Spatial variation).

The intra-individual variation was evaluated by comparison of the mean and variance of the results obtained from the different localizations. Provided the requirements for distribution and variance homogeneity of the samples was fulfilled, the means were compared by applying a Dunnett's test and the variation by means of an F-test. A two-way analysis of variance was applied to compare the variance between different localizations with the variance between corresponding localizations in different individuals from the same age group. The parameters obtained from similar localization on the right and left sides were compared by means of Student's t-test.

Influence of the staining procedure. The variation in the determination of osteoid due to different staining method was evaluated on the basis of a description of the distribution of the differences between the parameters obtained by Toluidin and by Mason trichrome staining. Fisher's regression line and a correlation coefficient were calculated.

RESULTS

The double determination of the Mason trichrome stained sections from 20 patients revealed that the method did not include any systematic errors (Table 1). The quantification procedure had a fairly high degree of precision (comp. I) and had only a slight influence on the results compared to the inter-individual variation (comp. II).

Variation in the histomorphometric data (AVTB, OS, OV and RS) due to differences in the localization or orientation were calculated the results appearing from Table 2. It was shown that the results of the quantification were not dependent on the orientation, whereas a deviation from the standard localization on more than 2 cm downward ($p < 0.10$) or backward ($p < 0.10$, $p < 0.05$) implied differences in the amount of trabecular bone. The two-way analysis of variance confirmed this result, as the variance component due to localizations was significantly greater (82.87 %) than that between the corresponding localizations from normal individuals of the same age. The variation in the osteoid parameters was to be less dependent on the localization, as only parameters obtained from the summit (No. 6, Fig. 1) of the iliac crest and 4 cm below (No. 9, Fig. 1) exhibited a result that deviated slightly but not significantly ($p > 0.05$) from that obtained at the standard localization. No deviations were found in the resorption parameters between the different localizations.

Furthermore, there were no differences between the quantification values obtained on contra-lateral sides (Table 4).

Comparison of osteoid parameters (Table 3) showed that more surfaces were estimated as osteoid seams using Mason trichrome than with toluidin blue. Highly significant corre-

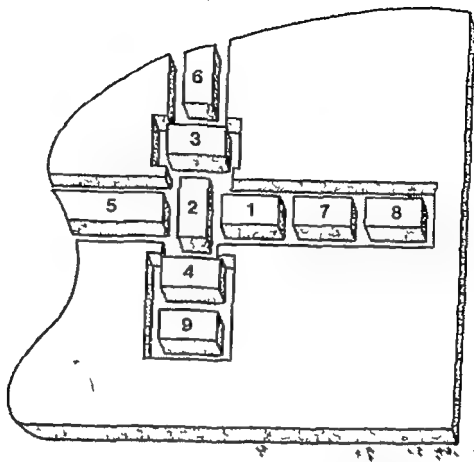


Fig 1 Tissue block from the iliac bone divided into minor bone blocks.

from adjacent areas. Thus, as well as an analysis of the influence on the volume of the orientation of the section, is described in the present study. The error included in the method will be calculated on the basis of double determination on the same section and the influence of different staining methods are evaluated on adjacent sections.

MATERIAL AND METHODS

The material for this study comprised tissue blocks from the right and left iliac crests removed at autopsy from 20 individuals between 20 and 40 years of age who were included in the normal material described elsewhere (Melsen *et al* 1978). Further iliac crest biopsies from 20 patients suffer-

ing from hyperthyroidism were included in the study.

The following parameters were measured: the amount of trabecular bone (AVTB), the extent, amount and width (OS OV WOS) of osteoid, osteoclastic resorption surfaces (RS) and the mean size of perivascular lacunae (POL) (Melsen *et al* 1978). With the purpose of studying the error included in the histomorphometry double determinations of sections from the above-mentioned normal individuals were performed at intervals of 3-4 months.

In order to study the influence of the localization of the biopsy and of the orientation of the section on the results of the quantitative analysis, 10 tissue blocks removed at autopsy were divided into nine minor transcortical bone blocks (1 cm wide and 0.5 cm high). Fig 1 illustrates the areas to be examined.

One block (No 2 Fig 1) comprised exactly the

error can thus be expected to be less than 1%.

In the present study the errors found in the determination of volume were slightly larger than what could be expected on the basis of the mathematical hypothesis but smaller than the error described by Ellis and Pearl (1973) and Huffer et al. (1973). These authors determined the reproducibility on bone from patients suffering from chronic renal failure. The deviation in the present study from the mathematically expected could partly be ascribed to the fact that the border between mineralized bone and osteoid as well as between osteoid and the surrounding tissue can be more or less distinct. Another explanation could be that the trabeculae are not completely randomly distributed, a fact which is a prerequisite for the calculation. The difference between the double determinations was, however, in no case of the same magnitude as the differences in measurements obtained from different individuals in the same age group and thus only of minor importance in the diagnosis of deviations from normality. Courpron (1972) compared measurements obtained by means of the integrating eye piece with similar values produced by means of an automatic scanning device (Quantimet) and found in the AVTB a mean difference of the same magnitude as between our double measurements. This implies that the variation is more likely a result of indistinct surfaces than of the distribution of the bone trabeculae.

The surface measurements, which were made according to Frost and Villanueva (1960) and Bordier et al. (1964) revealed that the error included in the measurements was, as could be expected, slightly larger than the one that could be calculated. This was especially the case for the osteoid surfaces and could thus again be ascribed to the indistinctness of the bone limits adjacent to the osteoid surface.

A comparison of the results obtained from differences in orientation and in localization showed that the orientation is of no importance for the determination of any of the

parameters, and thus supports the statements mentioned above. This finding is of great value as the orientation of a biopsy cylinder is difficult.

The findings on the different localizations corroborated those of Beck and Nordin (1960), Rasmussen and Bordier (1964), Garner and Ball (1966) and Gironx et al. (1975) who found no differences between adjacent biopsies from the iliac crest. Sections originating from areas more than 11 cm dorsally and inferiorly however showed trabecular bone volume different from that obtained from the standard localization. Gironx et al. (1975) found similar results comparing sections from different levels below the summit of the iliac crest. Compared with the vertebral biopsy as practised by Beck and Nordin (1960), Schenk and Verz (1969) and Blake and Sissons (1969), the localization chosen as a standard in this study appeared to have lower variation in trabecular bone volume among individuals of the same age and sex, whereas the mean values did not deviate. The same tendency was shown by Courpron (1972).

Differences between results obtained on contra-lateral sides have previously been analyzed by Rutz et al. (1973). Gironx et al. (1975) who, like the present authors, stated that no significant differences existed between the right and the left sides.

The influence of the staining on the evaluation of the volume of bone has previously been described by Courpron (1972) and Gironx et al. (1975) who both found a reduced amount in solochrome-stained compared with osteochrome-stained sections as prepared by Frost and Villanueva (1969). Gironx et al. (1975) compared solochrome cyanine with von Kossa stain and found no difference in the trabecular bone volume.

Compared with other methods of determining the amount of trabecular bone volume such as determination of density (Trotter and Petersen 1960) or radiodensity (Nordin et al. 1966, Melsen and Melsen 1975) the quantitative histological method has proved to be equally good, whereas photonabsorptometry

TABLE 1 *Double Determination of the Same Sections with an Interval of Several Months*

	d	s.d.	s.e.	s(i)	t	comp I	comp. II	F
AVTB	— .375	2.32	.52	1.66	25.07	8 %	92 %	24.42
OS	— .450	2.08	.47	1.47	14.08	7 %	93 %	29.00
OV	.040	.58	.13	.40	1.89	12 %	88 %	14.89
RS	.065	.70	.16	.48	3.59	30 %	70 %	3.46
POL	— .365	2.11	.47	1.48	51.11	33 %	67 %	5.09
WOS	— .035	0.12	.42	1.01	9.80	18 %	82 %	10.51

TABLE 2 *Comparison of Determinations of Histomorphometric Data from Different Localizations of the iliac crest*

i	AVTB			OS			OV			RS		
	\bar{x}	s.e.	d(2-s)	\bar{x}	s.e.	d(2-s)	\bar{x}	s.e.	d(2-s)	\bar{x}	s.e.	d(2-s)
2	24.57	1.59		17.13	1.87		2.02	0.38		4.26	0.24	
1	24.44	1.46	0.13	16.46	1.49	-0.67	2.17	0.30	0.15	4.00	0.18	0.26
3	24.96	1.42	0.39	16.30	2.11	-0.87	2.14	0.40	0.12	4.51	0.28	0.25
4	24.42	1.27	0.15	15.82	1.51	-1.31	1.74	0.34	-0.28	4.27	0.26	0.01
5	29.21	2.23	4.64*	16.20	1.53	-0.26	2.04	0.29	0.02	4.71	0.18	0.45
6	24.99	2.01	0.42	20.21	1.64	+3.08	3.07	0.37	1.03	4.40	0.23	0.14
7	20.39	1.57	4.18	15.83	1.60	-1.28	1.81	0.29	-0.21	3.78	0.24	-0.48
8	16.91	1.60	7.66**	16.83	1.49	-0.30	1.97	0.40	-0.05	3.87	0.25	-0.39
9	19.81	1.59	4.76*	13.43	1.92	-3.70	1.43	0.27	-0.59	4.44	0.40	0.18

** = 5 per cent significance * = 10 per cent significance according to Dunnett's test.

TABLE 3 *Difference in Determination of osteoid Parameters with Two Different Stains (Masson Trichrome-Toluidin blue)*

	\bar{d}	s.d.	s.e.	s(i)	t	corr r	regression line
OS	4.13	3.17	.71	3.65	5.81**	.96***	$y = 1.19x - 1.23$
OV	.22	1.04	.25	.73	0.30	.87***	$y = 1.22x - 0.65$

** $P < 0.05$

*** $P < 0.01$

TABLE 4 *Difference in Bone Parameters obtained from the Right and Left Side of the Iliac Crest*

Variable	\bar{d}	s.d.	s.e.
AVTB	0.48	1.86	0.59
OS	-0.67	3.29	0.25
OV	-0.37	0.42	0.16
RS	-0.10	0.55	0.20

lations were however found between the two methods.

DISCUSSION

The error involved in the applied point count method which was first described by Delesse (1847) can be mathematically calculated as a variable depending on the number of points counted. Corresponding to 1600 points, the

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as introduced by *Cameron and Sorensen* (1963) is more accurate in determining the mineral content in bones (BMC). As the inter individual variation in the degree of mineralization is of the same magnitude as the inter-individual variation in bone volume (*Robinson* 1960) determined histologically the photonabsorption method does not *per se* entail any advantage in the diagnostic procedure but may be a valuable supplement, especially in longitudinal studies of changes in the amount of bone in the individual patient.

The histomorphometric methods further more yield information on parameters related to bone turnover which cannot be obtained by other methods. These parameters seem hardly to be dependent on localizations, making it possible to obtain continuous information through consecutive biopsies with certain intervals.

It appeared that the result of the measurements of the osteoid surfaces was slightly influenced by the staining method. The difference was, however, less than the standard deviation within the individual age groups and thus has only an influence in border line cases.

Osteoid parameters have been studied by *Huffer et al* (1975) who compared osteoid determined in hematoxyline eosine stained decalcified and von Kossa stained undecalcified sections and found no difference likewise *Aleuier and Edouard* (1973) found no difference when comparing osteoid values obtained from von Kossa and solochrome stained undecalcified sections.

It is of importance to notice that the value of the bone biopsy as a diagnostic tool is based not on the information obtained from the individual parameters, but on the combination of the changes observed (*Schenk and Mierz* 1967). The present study has shown that none of the errors included in the method are of such a magnitude as to bias the evaluation of a biopsy.

This study was supported by The Danish State Research Foundation (Grant No. 512-1719 512 5132).

We are very grateful to The Institute of Forensic Medicine, University of Aarhus and The Institute of Pathology Hospital of Holstebro, for much help in the collection of material.

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the parameters expressing the amount, structure or remodelling of bone it is of the utmost importance to select a reference material very critically avoiding possible influences of previous or recent diseases. As immobilisation will lead to a shift in the parameters reflecting the bone turnover and mass (Albright 1941 Landry and Fleisch 1964 Donaldson 1970, Lignon *et al.* 1970 Kriensen and Aronson 1974 Elchler 1970 Minaret 1973) it is also important to exclude material obtained from autopsies of previously healthy individuals who prior to death, have been immobilized. The optimum reference material would, therefore, without any doubt be a biopsy material from healthy carefully examined individuals representing different sex and age groups.

Inter racial differences have been described (Trotter *et al.* 1960) whereas little is known about intra-racial variation. Such variations might be caused by various external factors as dietary vitamin D, Calcium intake and exposure to sunshine (Aron *et al.* 1974 Nielsen and Vosekilde 1975 Mosekilde and Nielsen 1976) for which reason it is necessary to establish a normal material in different geographical areas.

In order to increase the diagnostic value of bone biopsies, the aim was to establish the normal values, mean and range, in a Danish population, for the various quantities included in a histomorphometric analysis.

MATERIAL AND METHODS

The material selected for the study included necropsy material as well as biopsy material.

The necropsy material consisted of tissue blocks from the iliac crest removed at autopsy from 105 individuals. The square tissue blocks, including the anterior superior spine, were obtained by sawing 6 cm dorsal to the spine and 6 cm below the summit (Melsen *et al.* 1978).

The following criteria should be fulfilled in order to accept the individual to be included in the material

- 1) Sudden, unexpected death, preferably due to accidents or coronary occlusion.
- 2) No immobilisation or hospitalization before death.

- 3) No congenital diseases.
- 4) No information in the case history of endocrine or metabolic disorders.
- 5) No autopsy diagnosis apart from a "normal" degree of atherosclerosis and signs of senile atrophy.

The biopsy material comprised bone cylinders from 30 persons whose haemoglobin concentration, blood sedimentation rate, thyroid-, parathyroid-, kidney- and liverfunctions were normal. The subjects received tetracycline (600 mg dimethylchlor tetracycline on day III + 12 and on day 4 + 3) before biopsy in order to obtain double labelling of bone for further studies of dynamic parameters (Melsen and Mosekilde to be published).

The biopsies were removed in local anaesthesia with a thiopine (Bordier *et al.* 1964) from a standardized location 2 cm behind and 2 cm below the anterior superior iliac spine.

The sex and age distribution of the total material appears from Fig. 1.

The necropsies as well as the biopsies were fixed for at least 3 days in concentrated methanol immediately after removal of the tissue block. In the necropsies a bone block comprising exactly the area where the bone biopsies normally are taken was removed shortly after fixation. These small bone blocks as well as the biopsies were embedded in methylmethacrylate as described by Jewury (1963). Following the embedding, 7 μ thick serial undecalcified sections, were cut on a Jung microtome model K. (Melsen *et al.* 1978).

The tissue blocks from the necropsy material were serial sectioned all the way through and all sections left in jars containing 70 per cent alcohol, whereas only the sections from the middle third of the biopsy cylinders were stored. From each jar 12 sections were selected randomly and stained with Masson trichrome (for determination of bone mass and osteoid parameters) and Goldner trichrome (for determination of osteoclastic resorption surfaces). From an adjacent area a tissue block was removed, decalcified in 5% nitric acid, and 4 microns thick van Gieson stained histological sections were produced (for measuring the size of perivascular lacunae).

To evaluate the representativity of the necropsy material the histomorphometric data obtained from 10 biopsies were compared with those from necropsies of individuals with the same age and sex distribution.

The following parameters were obtained by measuring or calculating:

1. Trabecular Bone (Fig. 2a-2d)

- 1) Absolute volume of trabecular bone (AVTB) (Delesee 1947 Bordier *et al.* 1964): The percentage of a given volume of iliac bone, ex-

HISTOMORPHOMETRIC ANALYSIS OF NORMAL BONE FROM THE ILIAC CREST

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F Meisen B Meisen, L. Mosekilde and S Bergmann Histomorphometric analysis of normal bone from the iliac crest. Acta path. microbiol. scand. Sect. A 86 70-81 1978

Bone specimens from a standardized area of the iliac crest were obtained at autopsy in 105 individuals after sudden unexpected death and at biopsy in 30 living volunteers. Seven micron thick sections of the plastic embedded undecalcified material were produced and stained. The amount of cancellous and cortical bone and the parameters attached to description of bone remodelling were determined by point counting and simple measurements, in order to establish normal mean values and range in a Danish population. A decrease in the amount of bone, trabecular as well as cortical was found with increasing age in both males and females. The extent, volume and width of osteoid seams and the osteoclastic resorption were found to be age-independent. The osteoclastic resorption in cortical bone in males decreased however with increasing age. The reported values are of the greatest importance for the use of histomorphometric analysis of bone biopsies as a diagnostic tool in metabolic bone diseases. It is stressed, however that supplementary information of the dynamic aspects of the bone remodelling would be necessary for the understanding of the pathogenesis of bone changes. Such information might be obtained by using tetracycline double labelling

Key words Iliac crest bone structure histomorphometric studies.

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Received 20.7.77 Accepted 12.8.77

With an increasing understanding of bone as a sensitive tissue reflecting calcium homeostasis (Frost 1960 1961 1963 Bordier and Tun-Chot 1972 Rasmussen and Bordier 1974 Jaworski 1972 Rutz *et al* 1973 Hufnagel 1974 Huffer 1975) it has become of great value to recognize even slight deviations in any of the parameters attached to the description of bone.

Until recently these changes have been observed mainly in parameters related to the turnover of the cancellous bone (Garner and Ball 1966 Harris and Heaney 1969 Nordin

et al 1966 Delling 1975) It has, however been established that cortical bone responds just as readily to homeostatic deviations as trabecular bone (Meunier *et al* 1972 Meisen and Mosekilde 1977 Frost 1963 Epker *et al* 1964 Olah 1974 Jaworski *et al* 1972)

In the evaluation of the various measurable parameters it is, therefore, of the greatest importance to determine normal values for the parameters as they occur in normal individuals of different age and sex, in cancellous as well as in cortical bone. Well knowing that even slight variations in calcium phosphorus homeostasis may result in changes in

al. 1971) The percentage of trabecular bone volume occupied by osteoid. It was measured by the point counting, using a Zeiss integrating filter with 100 points at $160\times$ magnification. 400 points projected on bone or osteoid were counted.

- 4) *Mean width of the osteoid seams (WOS)* was measured with micrometric eyepiece as a mean of 4 extreme measurements in all osteoid covered surfaces. The osteoid seams were identified at $63\times$ magnification and measured at $400\times$ magnification.
- 5) *Relative volume of osteoid (AOV)* was calculated as $\frac{OV \times AVTB}{100}$
- 6) *Trabecular osteoclastic resorption surfaces (RS)* (Boraher et al. 1964) were measured as active as well as inactive resorption surfaces as a per cent of total trabecular bone surfaces. The resorption surfaces were identified as scalloped interruptions of the trabecular lamellar structure, with or without osteoclasts. The reading was performed in the same way as OS.

1 Cortical Bone

- 1) *Mean cortical width (MCW)* was calculated as the mean of 10 measurements on each cortical lamella, using conventional micrometric eyepiece and a reference plate.
- 2) *Porosity (POR)* The percentage of cortical bone area occupied by canals (Haversian and Volkmann canals). It was determined by point counting in 50 randomly selected fields, using the 100 points Zeiss integrating filter.
- 3) *The index of canals occupied by osteoclastic resorption was expressed as per cent of the total number of canals (CAR)*.
- 4) *Mean size of perinuclear lacunae (POL)* (Mennert et al. 1971) was expressed as the mean product of length and width of 50 randomly selected lacunae measured with micrometric eyepiece.

Statistic

A statistical description of the above data obtained was carried out for each age group and for each sex. Furthermore, two-way analysis of variance model I (Sokal and Rohlf 1969) was performed with the purpose of comparing the variations within and between age groups.

In the case of significant difference between the age groups, regression line was constructed, and its inclination was compared to 0. In the case of no significant difference between age groups, the different groups were pooled, and description of the distribution according to kurtosis and skewness was given. Furthermore the parameters obtained in

the two sexes were compared in the case of fulfillment of normality with a Student's *t* test, otherwise with a Mann-Whitney U-test.

Statistics for evaluation of the reproducibility and error of the method will be discussed in following paper (Stelsen et al. 1978).

RESULTS

The results of the quantitation carried out on bone specimens from normal individuals appear in Tables 1-6. The column to the far right shows the result of the analysis of variance (F).

Amount and structure of bone Table 1 gives the amount of trabecular bone (AVTB) within different sex and age groups. A regression analysis shows a significant fall in the amount of bone with increasing age (Table 1 and Fig. 3) both in males and in females. The amount of trabecular bone was in the youngest group higher in females than in males, whereas the more severe bone loss in women between the age of 50-59 resulted in an equal amount of trabecular bone in the two sexes in old age.

The values for mean cortical width (MCW) and cortical porosity (POR) are shown in Table 4 a, b. These parameters of cortical bone mass revealed that there is a gradual loss of cortical bone with increasing age. This loss was expressed both by a decrease in MCW and in the females by an increase in POR.

Amount and shape of osteoid Table 2 a, b and 5 gives the amount, width and extension of osteoid seams. The measurement of osteoid covered surfaces (OS) revealed no significant sex-difference or change with increasing age. The relative osteoid volume (OV) decreased slightly but insignificantly with increasing age. The decrease in absolute volume of osteoid (AOV) with increasing age was more pronounced as the amount of trabecular bone decreased with increasing age. No significant sex-difference was found in OV or AOV.

The mean width of the osteoid seams (WOS) showed only slight variations between age and sex groups.

Bone resorption The measurements of the

MALES

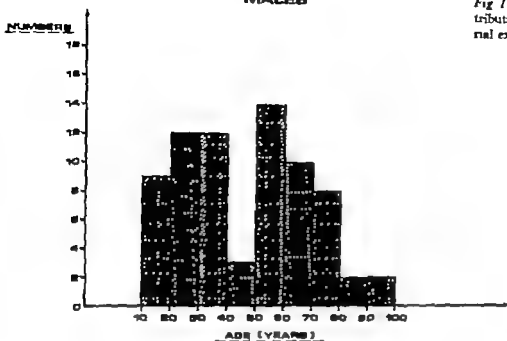
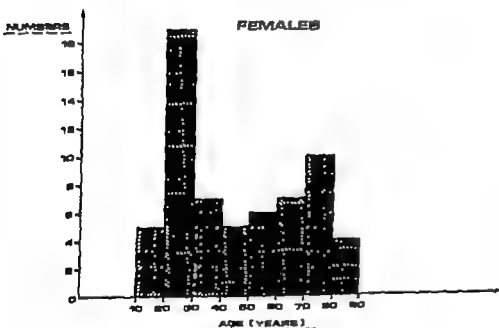


Fig 1 The sex and age distribution of the total material examined.

FEMALES



cluding medullary or vascular spaces but including mineralized as well as osteoid tissue. It was measured at a 25 x magnification by means of point counting, using a Zeiss I integrating eyepiece containing 25 points. 64 fields selected at random from the 12 sections were examined corresponding to 1600 testing points.

- 2) *Relative osteoid surface (OS)* The bone surface covered by osteoid expressed as a per

centage of the total trabecular bone surfaces. The reading was performed at a 65 x magnification, using a Zeiss integrating eyepiece containing a grid with six segments. 400 intersections between segments and trabecular bone surfaces were counted. The number of intersections between segments and osteoid covered surfaces was expressed as a per cent of the total number of intersections.

- 3) *Relative osteoid volume (OI)* (Courpron et

al 1971). The percentage of trabecular bone volume occupied by osteoid. It was measured by the point counting, using a Zeiss integrating filter with 100 points at a 160 \times magnification. 400 points projected on bone or osteoid were counted.

- 4) *Mean width of the osteoid seams (WOS)* was measured with a micrometric eyepiece at mean of 4 extreme measurements in all osteoid covered surfaces. The osteoid seams were identified at 63 \times magnification and measured at 400 \times magnification.
- 5) *Absolute volume of osteoid (AOV)* was calculated as
$$\frac{OV \times AVTB}{100}$$
- 6) *Trabecular osteoclastic resorption surfaces (RS)* (Border \equiv al. 1964) were measured as active as well as inactive resorption surfaces \equiv a per cent of total trabecular bone surfaces. The resorption surfaces were identified as scalloped interruptions of the trabecular lamellar structure, with or without osteoclasts. The reading was performed in the same way in OS.

2. Cortical Bone

- 1) *Mean cortical width (MCW)* was calculated as the mean of 10 measurements on each cortical lamella, using conventional micrometric eyepiece and a reference plate.
- 2) *Porosity (POR)* The percentage of cortical bone area occupied by canals (Haversian and Volkmann canals). It was determined by point counting in 30 randomly selected fields, using the 100 points Zeiss integrating filter.
- 3) *The number of canals occupied by osteoclastic resorption* was expressed as a per cent of the total number of canals (CAR).
- 4) *Mean size of perivascular lacunae (POL)* (Stern *et al.* 1971) was expressed as the mean product of length and width of 50 randomly selected lacunae measured with a micrometric eyepiece.

Statistics

A statistical description of the various data obtained was carried out for each age group and for each sex. Furthermore two-way analysis of variance model I (Sokal and Rohlf 1969) was performed with the purpose of comparing the parameters within and between age groups.

In the case of a significant difference between the age groups a regression line was constructed, and its inclination was compared to 0. In the case of no significant difference between age groups, the different groups were pooled, and a description of the distribution according to kurtosis and skewness was given. Furthermore the parameters obtained in

the two sexes were compared in the case of fulfilment of normality with a Student's *t*-test, otherwise with a Mann Whitney U-test.

Statistics for evaluation of the reproducibility and error of the method will be discussed in following paper (Stern *et al.* 1978).

RESULTS

The results of the quantitation carried out on bone specimens from normal individuals appear in Tables 1-6. The column to the far right shows the result of the analysis of variance (F).

Amount and structure of bone Table 1 gives the amount of trabecular bone (AVTB) within different sex and age groups. A regression analysis shows a significant fall in the amount of bone with increasing age (Table 1 and Fig. 3) both in males and in females. The amount of trabecular bone was in the youngest group higher in females than in males, whereas the more severe bone-loss in women between the age of 50-59 resulted in an equal amount of trabecular bone in the two sexes in old age.

The values for mean cortical width (MCW) and cortical porosity (POR) are shown in Table 4 a, b. These parameters of cortical bone mass revealed that there is a gradual loss of cortical bone with increasing age. This loss was expressed both by a decrease in MCW and in the females by an increase in POR.

Amount and shape of osteoid Table 2 a, b and 5 gives the amount, width and extension of osteoid seams. The measurement of osteoid covered surfaces (OS) revealed no significant sex-difference or change with increasing age. The relative osteoid volume (OV) decreased slightly but insignificantly with increasing age. The decrease in absolute volume of osteoid (AOV) with increasing age was more pronounced as the amount of trabecular bone decreased with increasing age. No significant sex-difference was found in OV or AOV.

The mean width of the osteoid seams (WOS) showed only slight variations between age and sex groups.

Bone resorption The measurements of the

TABLE 1 *Absolute Volume of Trabecular bone (AVTB) in Relation to Age and Sex*

Age		10-19	20-29	30-39	40-49	50-59	60-69	70-79	> 80	F
Males	N	9	12	12	3	14	10	8	4	
	\bar{x}	24.49	24.55	22.92	17.37	20.41	13.74	16.24	13.35	1.47
	SE	1.74	2.28	1.05	1.88	1.24	1.24	2.39	2.79	
Females	N	5	19	7	5	11	7	10	4	
	\bar{x}	31.78	26.76	26.49	22.54	20.10	15.49	16.97	17.65	1.48
	SE	2.70	1.70	2.35	3.50	2.40	1.77	1.96	3.24	

TABLE 2a. *Osteoid Parameters in Relation to Age from Males*

Age		10-19	20-29	30-39	40-49	50-59	60-69	70-79	> 80	F
Osteoid surface (OS)	N	9	12	12	3	14	10	7	4	
	\bar{x}	17.82	18.87	14.89	17.23	20.34	16.04	18.30	11.95	0.99
	SE	2.80	2.52	2.18	5.48	1.22	2.38	2.51	3.17	
Osteoid volume (OV)	N	9	12	12	3	14	10	7	4	
	\bar{x}	2.58	2.52	1.77	2.17	3.04	1.62	1.83	0.90	1.94
	SE	0.56	0.32	0.28	0.90	0.53	0.31	0.50	0.20	
Absolute osteoid volume (OV abs.)	N	9	12	12	3	14	10	7	4	
	\bar{x}	0.65	0.68	0.37	0.40	0.65	0.22	0.31	0.12	0.61
	SE	0.17	0.10	0.06	0.20	0.13	0.04	0.10	0.02	
Width of osteoid seam (WOS)	N	6	8	7	3	10	4	4	1	
	\bar{x}	10.88	10.38	9.84	9.13	10.63	10.03	9.56	6.50	0.41
	SE	0.77	0.76	0.68	1.63	1.00	1.11	2.69	0	

TABLE 2b. *Osteoid Parameters in Relation to Age from Males*

Age		10-19	20-29	30-39	40-49	50-59	60-69	70-79	> 80	F
Osteoid surface (OS)	N	5	19	7	5	11	7	10	4	
	\bar{x}	14.66	16.67	14.11	14.08	19.32	19.79	18.20	12.70	1.24
	SE	1.89	1.59	2.60	1.72	3.06	3.40	3.40	0.80	
Osteoid volume (OV)	N	5	19	7	5	6	7	10	4	
	\bar{x}	2.10	2.39	1.71	1.32	1.90	2.04	2.10	1.53	0.50
	SE	0.67	0.39	0.44	0.51	0.35	0.60	0.47	0.21	
Absolute osteoid volume (OV abs.)	N	5	19	7	5	6	7	10	4	
	\bar{x}	0.62	0.65	0.46	0.31	0.41	0.35	0.34	0.23	1.34
	SE	0.15	0.13	0.13	0.12	0.12	0.11	0.08	0.06	
Width of osteoid seam (WOS)	N	3	18	7	5	6	6	8	3	
	\bar{x}	10.43	9.74	8.29	7.98	9.48	9.75	8.15	8.97	1.16
	SE	2.26	0.37	1.04	0.47	0.59	0.91	0.92	0.33	

TABLE 3. *Trabecular Resorption Surfaces (RS) in Relation to Sex and Age*

Age		10-19	20-29	30-39	40-49	50-59	60-69	70-79	> 80	F
Males	N	9	12	11	3	11	10	8	4	1.72
	\bar{X}	3.89	3.96	3.69	4.10	4.04	4.13	4.99	5.98	
	SE	0.38	0.49	0.25	0.83	0.29	0.61	0.56	0.14	
Females	N	5	18	7	5	6	7	10	4	0.99
	\bar{X}	3.22	3.67	3.99	3.36	3.88	3.63	4.26	3.70	
	SE	0.31	0.34	0.39	0.23	0.56	0.37	0.53	0.57	

TABLE 4a. *Cortical Parameter from Males in Relation to Age*

Age		10-29	30-49	50-69	> 70	F
Mean age		20.8	35.2	57.9	78.3	
Mean cortical width (mm) MCW	N	13	11	14	7	2.91
	\bar{X}	1217	1081	860	943	
	SE	106	100	73	118	
Porosity per cent POR	N	11	10	13	6	0.81
	\bar{X}	6.9	6.0	7.0	6.2	
	SE	1.0	0.5	0.7	1.11	
Cortical width active resorption CAR	N	11	10	12	7	3.34
	\bar{X}	4.3	1.8	1.7	1.3	
	SE	1.2	0.5	0.5	0.48	

TABLE 4b. *Cortical Parameter from Females in Relation to Age*

Age		10-29	30-49	50-69	> 70	F
Mean age		22.8	39.4	59.6	78.3	
Mean cortical width (mm) MCW	N	9	10	11	10	4.10
	\bar{X}	1606	1047	909	900	
	SE	276	134	98	105	
Porosity per cent POR	N	8	10	8	9	7.55
	\bar{X}	3.7	4.3	6.3	7.4	
	SE	0.9	0.2	0.6	0.8	
Cortical width active resorption CAR	N	8	10	11	10	0.82
	\bar{X}	2.1	1.8	2.2	3.0	
	SE	0.5	0.5	0.4	0.7	

extension of the trabecular osteoclastic resorption surfaces (RS) (Table 3.5) revealed an insignificant tendency towards an increase with increasing age. In females the resorption

surfaces seem to be more independent of age, apart from the age group 50-59 where a distinct increase in the resorption surfaces was found.

TABLE 5 *Distribution of the Pooled Parameters for Osteoid and Resorption*

		N	\bar{X}	S.D.	S.E.	G_1	G_2	KS-dmax
Males	OS	71	17.71	7.41	0.86	-0.07	-0.63	0.067
	OV	71	2.27	1.47	0.17	0.95	1.31	0.103
	WOS	43	10.15	2.60	0.38	0.97	1.86	0.097
	RS	70	4.18	1.42	0.17	0.65	-0.06	0.090
Females	OS	63	16.54	7.70	0.97	0.78	1.84	0.084
	OV	63	1.99	1.40	0.17	0.99	0.35	0.162
	WOS	36	9.14	2.76	0.37	0.15	3.29	0.079
	RS	62	3.92	1.35	0.17	0.37	-0.37	0.104

TABLE 6 *Corresponding Values Obtained from Autopsy and Biopsy Material*

		AVTB	OS	OV	RS
Autopsy	\bar{X}	25.9	13.7	1.9	3.8
	S.D.	7.4	6.1	1.3	0.8
Biopsy	\bar{X}	25.6	13.4	1.7	3.7
	S.D.	6.5	6.0	1.6	1.6

The relative number of canals in cortical bone with active resorption (CAR) (Table 4 a and 4 b) showed no sex-difference. A significant decrease with increasing age in males was mainly explained by a high value in the lowest age group ($y = -0.04x + 4.39$ $n = 40$ $r = -0.37$ $p < 0.05$).

The mean size of the periosteocytic lacunae (POL) was 51.2 ± 0.4 ($\bar{X} \pm SE$, $N = 25$). This parameter was not tested for variations according to sex and age.

A comparison between the results obtained from the autopsy and the biopsy material (Table 6) revealed no differences in the mass and remodelling of trabecular bone.

DISCUSSION

In the present study parameters describing bone mass and remodelling in cancellous and cortical bone in a normal Danish population have been determined and related to differences in age and sex. A comparison between the results obtained from the autopsy and the biopsy material revealed no difference and

thus confirmed that the autopsy material could be considered representative of a normal population.

The significant decrease with age in trabecular bone mass (AVTB) shown in this investigation is in agreement with findings by other authors who have studied bone changes in the iliac crest, whether the method was histomorphometry (Lordin *et al.* 1966, Schenk and Verz 1969, Meunier *et al.* 1973, Dellng 1973, Courpron 1972, Bordier and Tun-Chot 1972, Giroux *et al.* 1975, Dellng 1975) or quantitative microradiography (Ikematsu and Sissons 1969). Similar age related changes in the amount of cancellous bone have been demonstrated in vertebrae, ribs and sternal bone (Lindahl and Lindgren 1962, Caldwell 1962, Arnold *et al.* 1966, Atkinson 1967, Meunier *et al.* 1973, Giroux *et al.* 1975).

The difference shown in the amount of trabecular bone in the two sexes confirm the findings by Courpron (1972) that young females have a higher percentage of bone than males, and a more pronounced decrease in trabecular bone volume after the age of fifty.

Patients suffering from osteoporosis (Chalmers and Weaver 1966) and other conditions with osteopenia (Melsen and Nielsen 1977, Meunier *et al.* 1972) very often reveal spontaneous fractures in cancellous bone region (vertebrae and femoral neck). The natural question is, therefore, whether the amount of trabecular bone in iliac crest biopsies is representative of other cancellous bone regions in the skeleton. Dunnill *et al.* (1967) and

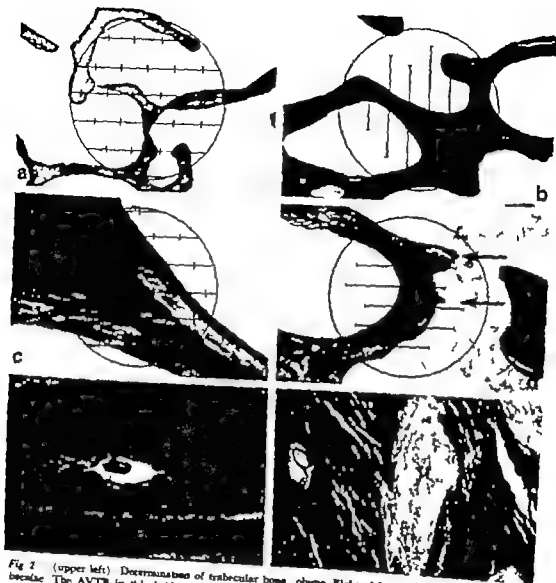
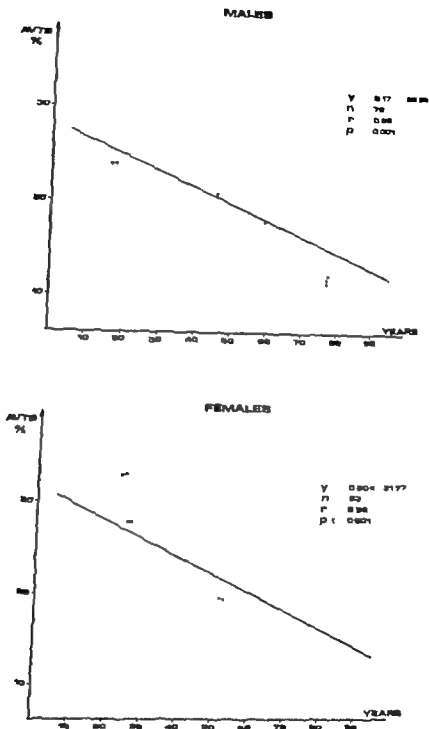


Fig 2 (upper left) Determination of trabecular bone volume. Eight of 23 points are projected on trabeculae. The AVTB in this field is 32 per cent (Goldner trichrome stain)
 b (upper right) Determination of osteoid covered surfaces. Three of 8 intersections are between osteoid covered surfaces and segments. The OS in this field is 37.5 per cent. (Masson trichrome stain)
 (middle left) Determination of relative osteoid volume. Nineteen points are projected on bone. Two points are on osteoid OV in this field is 10.5 per cent. (Masson trichrome stain)
 d (middle right) Two of nine intersections are between segments and a resorption lacuna (between the arrow.) The RS in this field is 22.2 per cent. (Goldner trichrome stain)
 (lower left) Measurement of periosteocytic larva as the product of length and width. (Decalcified Van Gieson stained section)
 f (lower right) Cortical canal with osteoclastic resorption to the left and osteoid to the right. (Goldner trichrome)

Fig 3 Correlation between AVTB in per cent and age in years in the two sexes.



Doyle (1972) claimed that a reliable prediction of the amount of bone in the individual axial skeleton could not be carried out on the basis of iliac crest measurements a statement which however does not exclude the existence of a correlation between the amount of trabecular bone or functional properties in the different parts of the skeleton (Iost 1963

Jowsey *et al* 1965 Chalmers and Hcarer 1966 Meunier 1968 Meunier *et al*, 1973 Adams *et al* 1970 Citroux *et al*, 1975 Melson *et al* 1976)

The amount of cortical bone as that of trabecular bone was found to decrease with age. This was caused by a decrease in the mean cortical width in both sexes and by an

increase in the porosity in the females with increasing age. The results confirm those of Courpron (1972) who explained the decrease in cortical width as a result of an expansion of the marrow space. The changes in cortical bone mass seem to be unrelated to the region of the skeleton (Arnold *et al* 1966 Olah and Schenk 1969 Dequeker *et al* 1971).

Increased cortical porosity has been demonstrated in hyperthyroidism on X rays (Meema and Meema 1972) by quantitative micro-radiography (Adams *et al* 1967) and by bone histomorphometry (Mennier *et al* 1972, Melser and Mosekilde 1977). To increase the diagnostic value of a bone biopsy it is therefore of importance to establish the normal variation of this parameter.

The amount, extension and width of the osteoid seams were in the present study unrelated to age and sex. Slight but insignificant variations occurred however. Similar small variations in the osteoid parameters have been shown by other authors (Hoods *et al* 1968, Mierz and Schenk 1970 Courpron 1972 Mennier *et al* 1973 Bordier and Tan-Chot 1973 Drilling 1975). The main tendency seems to be a fall in OS and OV in the first 4-5 decades, followed by a slight increase. A more pronounced decrease is observed in the absolute volume of osteoid (AOV) which can mainly be explained by a decrease in the amount of trabecular bone with increasing age.

The trabecular osteoclastic resorption surfaces (RS) were unrelated to sex and age, confirming the results of Courpron (1972) and Mennier *et al* (1973). A slight increase in RS was, however, found in the last decades. This finding is in accordance with the observations of Schenk *et al* (1972) Bordier and Tan-Chot (1973) and Joursey *et al* (1965).

The cortical osteoclastic resorption (CAR) increased with increasing age in males but was unrelated to age in the females. The increase in cortical porosity among the females with increasing age could not, therefore, be explained by an increase in CAR. The inter-relationship between CAR and porosity however has previously been demonstrated in

hyperthyroid patients (Melsen and Mosekilde 1977 Mosekilde *et al* 1977).

As the mean size of perosteocytic lacunae has been shown to be uninfluenced by age (Mennier *et al* 1971 Courpron 1972) this parameter was only determined on 25 randomly selected individuals, in order to establish mean and range within a Danish population.

The representativity of the iliac crest for other parts of the skeleton has mainly been based upon determinations of bone volume, mass or functional properties, whereas parameters reflecting bone remodelling have received only slight attention. Joursey *et al* (1965) have on microradiographs, however shown highly significant correlations between bone formation and resorption surfaces in the iliac crest and other parts of the skeleton. Furthermore, it has been shown that the parameters of bone mass and bone remodelling varies very little within different localizations close to the standard area for bone biopsy in the iliac crest (Melsen *et al* 1978).

The present investigation gives information about the normal values of the static parameters of bone i.e. the result of a remodelling process. No information is given about the rate of bone changes. Changes in the amount of osteoid may thus reflect variations in the rate of osteoid formation variations in the rate of mineralization, or combined variations. The dynamic of bone mineralization can be elucidated further by tetracycline double labeling of the surfaces active in mineralization (Frost 1969). Determination of the resorption surfaces, whether active or inactive, gives no information about the rate of bone resorption. Such information can only be obtained indirectly by determination of bone formation rate and bone mass.

This study was supported by The Danish Stat Research Foundation.

(Grant No. 512-1719 512 5152)

We are very grateful to The Institute of Forensic Medicine, University of Aarhus and The Institute of Pathology Hospital of Holstebro for much help in the collection of material.

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BRIEF REPORTS

DIPYRIDAMOLE AND CAPILLARY PROLIFERATION A PRELIMINARY REPORT

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Tornling Göran, Unger Gunnar, Ljungqvist Arne and Carlsson Sture. Dipyridamole and capillary proliferation. A preliminary report. Acta path. microbiol. scand. Sect. A, 86: 82, 1978

During dipyridamole treatment of rats cellular proliferation is observed in the myocardium and skeletal muscle. The proliferative activity in the myocardium is exclusively localized to capillary walls. The localization within the skeletal muscle has not been analyzed.

Key words: Dipyridamole, DNA-synthesis, heart, skeletal muscle.

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Received 31.x.77 Accepted 31.x.77

Dipyridamole (Persantin®) is a well-known vasodilator and clinical effects have been reported in the treatment of coronary heart disease (Roberts *et al.* 1968) and peripheral upper limb ischaemia (Widling & Flute 1974). Part of this effect has been ascribed to the development of anastomosing vessels (Fam *et al.* 1964, Roberts *et al.* 1968). This raises the question whether or not dipyridamole is capable of inducing growth of blood vessels.

We have investigated the effect of dipyridamole on cellular proliferation in the heart and skeletal muscle of Sprague-Dawley rats. One hundred and nine male and female animals of various ages received dipyridamole i.p. 3 mg per kilogram body weight twice a day over a period of 3 weeks. The animals were sacrificed 15 minutes after an i.v. injection of tritiated thymidine. The DNA synthesis was measured as c.p.m./ μ g DNA in a scintillation counter and the localization of the thymidine incorporation was assessed by autoradiography.

In comparison with control animals of the same ages the dipyridamole treated animals showed an

increased DNA-synthesis in cardiac ($p < 0.005$) as well as in skeletal muscle of the hind limb ($p < 0.0005$). All radioactivity in the hearts was located in the nuclei of capillary wall cells, indicating a capillary neoformation similar to that previously demonstrated in rats subjected to swimming exercise (Mandache *et al.* 1973, Ljungqvist & Unger 1973). It is likely that the increase in DNA-synthesis in skeletal muscle is also the result of a proliferation of capillary wall cells, although this has not yet been investigated by autoradiography.

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III SULPHATED BLOOD-GROUP SUBSTANCE IN PANCREAS A FACTOR IN CYSTIC FIBROSIS?

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Rølla, Gunnar Melsen, Birt and Sjøvåg, Torleif. Is sulphated blood-group in pancreas a factor in cystic fibrosis? Acta path. microbiol. scand. Sect. A, 86 83-86 1978.

The present study showed that the pancreas of two monkey species contained sulphated blood-group substance. Such strongly anionic molecules are known to bind calcium, and their solutions form gels in the presence of calcium. It is suggested that such a mechanism may be involved in cystic fibrosis.

Key words: Cystic fibrosis, sulphated glycoproteins.

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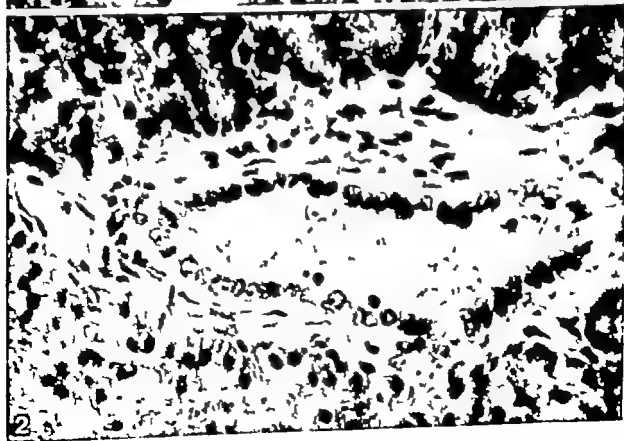
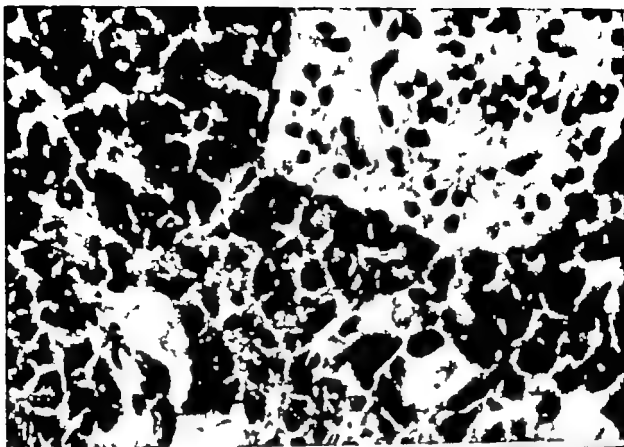
Received 12 x.77 Accepted 5.xl.77

Accumulation of calcium-rich materials has been suggested as the cause of the obstruction and enlargement of the pancreatic ducts in cystic fibrosis (CF) of the pancreas (Kjell et al. 1976). The nature of this material is not known.

The pancreas contains large quantities of ABO and blood-group material (Sjöström 1960, Lashley & Sherrinhan 1977). Some blood-group active glycoproteins are known to be sulphated (Yamamoto 1972, Sjøvåg & Rølla 1975, Woodhead & Desnick 1976) and thus strongly negatively charged. Such polyanions can bind calcium (Haug & Smidsrud 1970) and the addition of divalent cations to their solutions may result in gelation (Smidsrud 1974). We suspected these abnormal, over-sulphated, strongly anionic blood group substances might be involved in the obstruction of the pancreas in CF patients. Several reports indicate that the mucous glands of CF patients contain more sulphate (or more metachromatic material) than those of normal individuals (Lomb & Reid 1972, Wood et al. 1976).

In the present study we have used biochemical and autoradiographical techniques to determine whether sulphated blood group substance occurs in the normal pancreatic tissue of monkeys receiving radioactive sulphate. Previous studies have shown that radioactive sulphate is rapidly incorporated

into the epithelial glands of monkeys (Sjøvåg & Rølla 1974). We believe that a similarly rapid uptake may occur into the pancreatic tissue. Thus seven young monkeys of the species *Cercopithecus aethiops* and *Macaca fascicularis* were injected intraperitoneally with 1 mCi ^{35}S per kg body weight and were killed six hours later. One monkey of each species was perfused with glutaraldehyde, and the pancreatic glands removed, sectioned and processed for autoradiography. The technical procedures have been described elsewhere (Rølla, Melsen & Sjøvåg 1973). The remaining animals were exsanguinated, and the pancreatic glands were removed under sterile conditions, washed and incubated for 48 hours in Tyrodes salt solution at 37 °C. Similar preparations of salivary glands are known to release sulphated macromolecules into the supernatant fluid (Sjøvåg & Rølla 1974). The supernatants were then collected, heated for 20 min in boiling water (to inactivate enzymes) and to precipitate heat-labile proteins, centrifuged and dialysed for one week against several changes of distilled water. The supernatants were then concentrated by rotary evaporation to one eighth of the original volume, applied to a Bio-Gel A-0.5 m column, (1.6 x 40 cm) and eluted with 0.05 M phosphate buffer (pH 7). The eluate was monitored at 206 and 280 nm with a LKB 289 Ultracord recorder and the fractions (2 ml) were



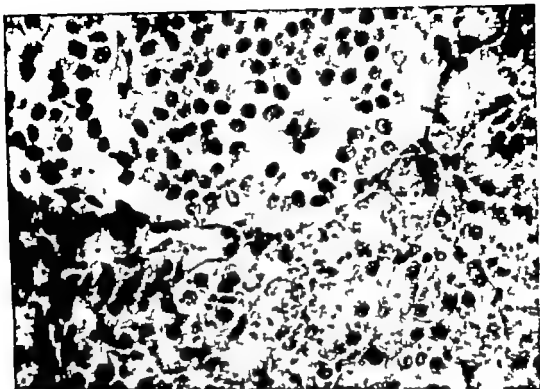


Fig 3 Photomicrograph in black and white corresponding to Fig. 1. For details, see above. Haematoxylin-eosin $\times 1000$.

tested for blood-group substance and virus haemagglutination inhibition activity by standard techniques as described previously (Sajó *et al.* 1974) and for sulphate by scintillation counting.

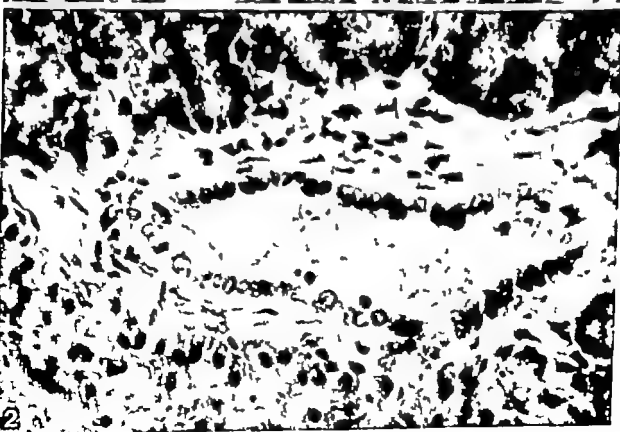
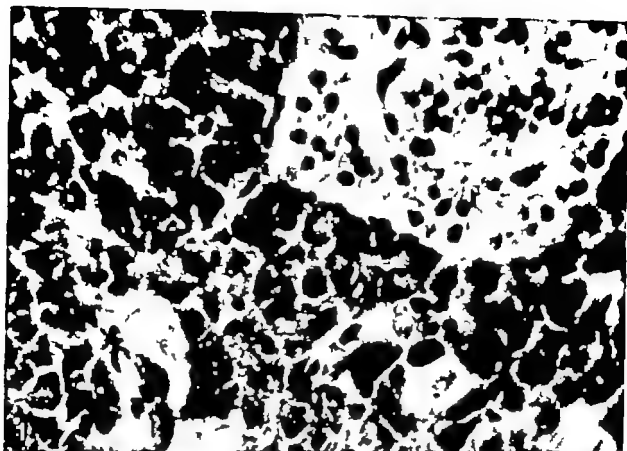
Autoradiography showed an even distribution of radioactivity over the acini, but almost none over the islets of Langerhans. Material inside the ducts was radioactive, indicating that the sulphated material was excreted into the ducts (Figs 1, 2 and 3).

A large peak of material which absorbs UV at 206 and 280 nm was eluted close to the void volume of the gel filtration column. This was followed by a lower and broader peak which was discarded. The large peak was highly radioactive and exhibited blood-group substance and virus haemagglutination inhibition activity the maxima of which coincided closely with the UV absorption (Fig. 4). This material was eluted as a single peak from a Sephadex DEAE column with a sodium chloride gradient from 0.1 M.

The results suggest that sulphated macromolecules are indeed produced in the monkey pancreas. Sulphate, blood-group substance and virus haemagglutination inhibition activity were eluted in the same peak. The homogeneity of the peak was tested in ion exchange chromatography and a similar purification procedure (heating, centrifugation and gel chromatography on Bio-Gel A-0.5m) gives homogeneous blood-group substance from whole sal. a., containing sulphate and exhibiting the same biological activities (Sajó & Rózsa 1975). Furthermore the distribution of sulphate over the exocrine part of the pancreas (Figs. 1, 2 and 3) coincides with the distribution of blood-group substances previously observed by fluorescence micro-

Fig 1 Photomicrograph of 5 μ m section of the pancreatic gland of *Cercopithecus aethiops* injected with 35 S. The grains over the glandular tissue are clearly visible. Few grains are seen over the islets of Langerhans (upper left). Haematoxylin and eosin (Stiles $\times 630$).

Fig 2 Photomicrograph from the same sample, showing duct containing material with grains, presumably sulphated blood-group active glycoprotein molecules. Numerous grains can be seen over the acini. Haematoxylin and eosin (Stiles $\times 630$).



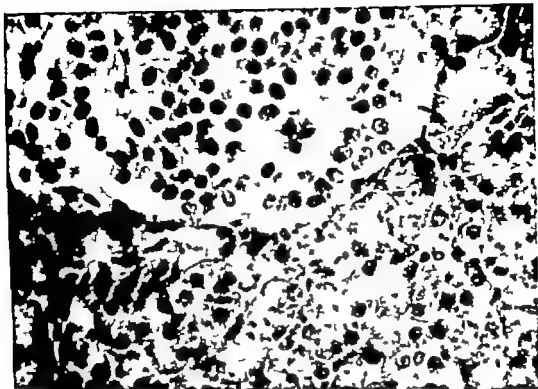


Fig 3 Photomicrograph in black and white corresponding to Fig 1. For details, see above. Haematoxylin and eosin $\times 1000$.

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Fig 1 Photomicrograph of a 5 μ m section of the pancreatic gland of *Corvus jelskii* injected with 35 S. The grains over the glandular tissue are clearly visible. Few grains are seen over the islets of Langerhans (upper right). Haematoxylin and eosin (Slide $\times 630$).

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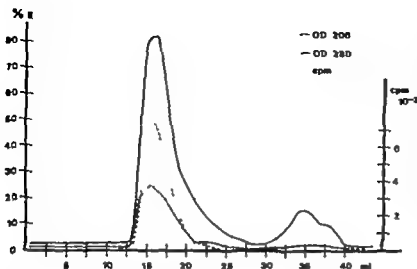


Fig 4 Elution diagram of an extract of the pancreatic gland of *Macaca fascicularis* on an agarose column (Bio-Gel A-0.5m). Sulphate blood-group substance activity and virus haemagglutination inhibition activity were found in the first peak, with maxima coinciding with the OD 206. Blue dextran (MW 2×10^6) was eluted in the same fraction. The abscissa gives fractions of 2 ml. For further details, see above.

copy (Sculman 1960 Ludvig & Sørensen 1977). It thus seems likely that sulphate and blood-group substance activity are associated with the same glycoprotein molecule.

The presence of sulphated blood-group substance in the pancreas of two species of primates strongly suggests that such material may also be present in the human pancreas.

One is tempted to wonder whether CF patients may produce more normal blood group substance than normal individuals or a blood-group substance with a higher sulphate content, and whether this material forms gel in presence of calcium.

It may be argued that CF patients exhibit symptoms in a variety of organs besides the pancreas and that nonsecretors of blood-group substance also exhibit CF. However lung secretions, sweat, bile-
semen, meconium and the secretions present in the upper gastrointestinal tract, all contain blood-group substances (see Watkins 1972) and it is likely that these are sulphated. It is thus conceivable that gel formation could occur at all these locations by interaction between abnormal sulphated blood-group substance and calcium ions. One single mechanism may thus explain all the symptoms encountered in cystic fibrosis. The saliva of non-secretors of blood-group substance contains sulphated molecules very similar to those occurring in the saliva of secretors (Sørensen & Rølla 1975). These molecules differ only in the nature of the terminal sugar residues. Such glycoproteins probably also occur in other exocrine products of non-secretors. Non secretors would thus be in the same situation as secretors as regards precipitation of sulphated proteins by calcium.

A high electrolyte concentration in sweat (Agnew *et al* 1953) and several other secretions is a well-known observation in cystic fibrosis. This may be a secondary effect (Hales 1958) reflecting bind-

ing of counterions to abnormal anionic material in the secretions.

The authors owe thanks to Dr L. Kornstad, National Institute of Public Health, Oslo, who performed the blood-group substance titrations of the monkey material and to Dr V. Jacobsen who helped with the tissue cultures.

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HIGHLY DIFFERENTIATED GASTRIC ADENOCARCINOMA ORIGINATING FROM THE NORMAL, NON METAPLASTIC GASTRIC EPITHELIUM

A Ultrastructural Study of a Case

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Teglbjærg, P Stubbø. Highly differentiated gastric adenocarcinoma originating from the normal, non-metaplastic gastric epithelium. An ultrastructural Study of a Case. Acta path. microbiol. scand. Sect. A, 86 87-89 1978

The first described case of a highly differentiated gastric adenocarcinoma, with light microscopic and ultrastructural features, pointing to the normal gastric epithelium as the site of origin, is presented

Key words: Gastric adenocarcinoma gastric epithelium ultrastructure.

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Received 23 ix.77 Accepted 1.xii.77

In later years, increasing importance has been attached to the role of intestinal metaplasia with regard to the histogenesis of gastric carcinoma (2, 4-5) so much so, that all highly differentiated adenocarcinomas (7) and also the majority of undifferentiated carcinomas (3) are considered as being derived from intestinal metaplastic epithelium. Ultrastructural studies have also emphasized the similarity between highly differentiated gastric adenocarcinomas and intestinal metaplastic epithelium (1, 8).

However *Malligen and Rembert* (8) published a histogenetic classification of gastric carcinomas and divided these tumours into 1) intestinal cell carcinoma, and 2) mucous cell carcinoma, which are, on the whole, analogous to *Laurén* (4) "intestinal-type carcinoma and diffuse carcinoma" and 3) pyloro-cardiac-gland carcinoma, the latter is not yet accepted internationally as a specific type of tumour.

In such circumstances it is considered of interest to publish the first highly differentiated gastric adenocarcinoma with histological and ultrastructural features which point to the normal, non-metaplastic gastric epithelium, possibly the pyloro-cardiac glands, as the site of origin.

Case History

A 69 year old man, who had complained of increasing dyspepsia for some 6 months.

On the 11/10-1976 total gastrectomy was performed after which, peritonitis developed and death occurred on the 21/1 1977. Post mortem examination was not carried out. The specimen for study was collected at the operating theatre.

The material for light microscopy was fixed immediately in 5% formalin and embedded in paraffin wax, cut and stained with haematoxylin-eosin and alcian-blue pH 2.5 - PAS. Nineteen blocks were removed for electron microscopy fixed immediately after removal in 5% glutaraldehyde in 0.2 M cacodylate buffer for 3 hours. After washing in the buffer solution, they were fixed in osmium tetroxide and embedded in epon. Thin (1 μ) sections were stained with toluidin blue. Ultra-thin sections (approximately 0.07 μ) were stained with uranyl acetate and lead acetate and examined in a Hitachi HS-8 electron microscope.

Material for electron microscopic comparison was taken from normal antral mucosa of another stomach removed owing to gastric carcinoma. This material was prepared in the same manner.



Fig 1 Section from the tumour. Neoplastic glands covered by a single layer of cylindrical epithelium, often with row of basally situated nuclei, light cytoplasm and distinct cell borders (haematoxylin-eosin $\times 400$)

Fig 2 The same area as in figure 1, showing muc granules in the luminal part of the cytoplasm of the tumour cells (alcian-blue pH 2.6 - PAS $\times 400$)

Fig 3 Electron microscopic picture of the tumour. The tumour cells which surround the lumen (L) have incomplete microvilli along the luminal border as well as number of secretory granules (X) of varying, often considerable electron density in the luminal part of the cytoplasm (compare with fig. 4) (E.M. $\times 3300$)

Fig 4 Electron microscopic picture from a normal pyloric gland. The cells of the epithelium, which surround the lumen of the gland (L) have complete microvilli along the luminal border and contain secretory granules (X) of varying, often considerable electron density in the luminal parts of the cytoplasm (E.M. $\times 3300$)

Pathology

The specimen consisted of the whole stomach. With its centre in the small curvature a 12×10 cm, large ulcerated tumour was observed, reaching from the cardiac region to the antrum.

Light microscopy. According to previously published light microscopic criteria (10) the present tumour was classified as pyloro-cardiac gland carcinoma. (Fig 1 and 2)

Electron microscopy. Electron microscopy showed highly differentiated adenocarcinoma. Incomplete microvilli could be observed in the luminal cytoplasmic membrane (Fig 3). Secretory granules were present in varying, often plentiful, amounts in the luminal part of the cytoplasm (Fig 3). The

granules were spherical, varying, but most often being of considerable electron density and measured 0.2 to 1.5μ . Identical, secretory granules were seen in the pyloric glands of normal non-neoplastic mucosa, removed from another stomach (Fig 4). Similarly the cells of these glands had incomplete microvilli. Mucin granules of the goblet cell type were not present in the tumour cells.

Discussion

The ultra-structure of the present tumour particularly the type of secretory granules, is almost identical with the ultra-structure of the normal cell of the pyloric glands or the normal, gastric surface epithelium, as described by Rabin et al. (9). The incomplete microvilli on the luminal surface of the tumour cells cannot be considered indicating intestinal histogenesis, inasmuch as these normally occur in the cells of pyloric glands (9). This ultra-structural study thus provides considerable evidence for concluding that the tumour has its origin in the normal, non-metaplastic gastric epithelium, possibly the pyloro-cardiac glands.

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PULMONARY EXCRETION OF CARBON BLACK INJECTED INTO THE CEREBRAL VENTRICLES OF THE RAT

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Bertheussen A. J., Diemer N. H., Præstholm, J. and Klinken, L. Pulmonary excretion of carbon black injected in the cerebral ventricles of the rat. *Acta path. microbiol. scand. Sect. A*, 86 90-92, 1978

After injection of a mixture of carbon black and a iodinated X-ray contrast medium into the cerebral ventricles of rats carbon black loaded macrophages were found in the alveolar septa in the lungs. It is concluded that contrast media used in cerebral ventriculography are eliminated at least partly by discharge of pulmonary macrophages.

Key words: Cerebral ventriculography, pulmonary excretion, macrophages.

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Received 24.xii.77 Accepted 24.xii.77

Pulmonary excretion of foreign bodies has been the subject of several studies (Aiscol & Bilbey 1958; Adlersberg *et al.* 1969).

After injection of a carbon black suspension into the bladder wall of rats, particles of carbon black are found in pulmonary macrophages about 24 hours later (Bertheussen & Nissen 1976). They assumed that an active pulmonary excretion of the carbon black particles took place via the macrophages.

Cerebral ventriculography showed Pantopaque® intracellularly in macrophages lying at the open dyme 1-4 months after the ventriculography (Gjerus Præstholm & Klinken 1976). Erythrocytes as well as inorganic/organic material in the spinal fluid disappear gradually. In what way and where this elimination takes place is not known.

It is the aim of the present study to investigate whether macrophages containing carbon black particles can be found in the lungs of rats subjected to cerebral ventriculography performed either with a contrast medium containing carbon black, or with a pure carbon black suspension.

MATERIAL AND METHODS

Methods of injection 1) In the first series 5 male rats (Wistar 450-490 g) were injected with a mixture of carbon black (Pelikan G11/1431a) and Ampaque® (300 mg I/ml). Of this mixture 0.03 ml was injected using the free-hand method of Klev & Præstholm (1974). Radiography immediately after the injection showed that the injected material was localised in the ventricular system (Fig. 1).

2) In a second series, 2 male rats (Wistar 210-240 g) were injected with a 3:1 mixture of physiologic saline and carbon black. At autopsy 30 days later small stains of carbon black were found in both series around the bore hole in the calvary and in the meninges corresponding to the site of injection. Furthermore, carbon black was found in the ventricular system, in the basal leptomeninges and in the brain tissue around the cannula track. As particles of carbon black may be transported from loose connective tissue, bone tissue and larger "open" vessels around the lesion it was decided to change the technique of injection in order to eliminate this source of error.

3) In this series of 6 male rats (Wistar 250 g) the carbon black suspension (same mixture as in 2) was injected through a catheter in the following way. In aether anaesthesia a midline incision was



Fig. 1 Ventriculogram approx. 2 minutes after injection of contrast-medium and carbon-black suspension showing contrast shadows in the ventricular system, cisterna magna and spinal canal

made across the skull. The periosteum was removed from an area of 5 mm in diameter. Two mm to the right of the sagittal suture and 1 mm behind the coronal suture 0.6 mm hole was made. At right angles to the surface of the skull a polyethylene catheter (Polystan PP 25) filled with heparin was inserted by 4 mm. The space between the bone and the catheter was closed with dental cement (Poly-Fix De Trey Frères) which also kept the catheter in place. The other closed end (15 cm) was bent at right angle and the midline incision was sutured above the catheter. Four days later this was reopened and 0.03 ml of carbon black/maline suspension (1:3) was easily injected through the catheter which was then sealed with hot forceps just above the skull. There was no escape of carbon black from the cranium via it in any of the cases.

Autopsy and histological method. Autopsy was performed after 3 days to 5 months in the series A and B, and after 4 (+41) days in series C. The brain, heart, and lungs were removed and fixed for

a further 4 days before they were dehydrated and embedded in paraffin. Lung tissue was cut on a serial microtome and stained with eosin for 1 min. Furthermore ordinary sections were made with haematoxylin-eosin and van Gieson staining.

Result

When using the polyethylene catheter method of injection, autopsy of the brains revealed no carbon black outside the leptomeninges. Carbon black in abundance was found in the ventricular system, in the subarachnoid space and especially around the foramina of Luschka and Magendie. In the brain a small amount of carbon black was found in macrophages surrounding the site of injection and in macrophages in the ventricular walls.

In the lungs of all rats macrophages containing carbon black were found in the alveolar septa. No macrophages were seen in the alveoles or in the bronchi (Fig. 2).

No particles of carbon black were found in the alveolar system of number of control animals.

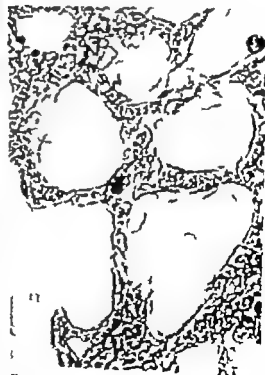


Fig. 2 Rat lung, 4 days after injection of carbon black in the ventricular system. A macrophage containing carbon black particles is seen in the alveolar septum. Eosin $\times 345$.

Discussion

In an earlier experimental work by *Bertheussen & Vissen* (1976) carbon black injected into the bladder wall of rats could be found in pulmonary macrophages. They suggested that the injected carbon black material was eliminated by an active pulmonary excretion via the macrophages. The present analogous study shows that carbon black injected into the central nervous system presents the same histological picture in the lungs. This may in fact be a more general way of reaction as earlier hypothesized by *Adlersberg et al* (1969). Only very few clinical experiments to this purpose have been performed.

Pulmonary elimination by means of macrophages may have etiologic significance in the development of e.g. lung damage after burns ("shock lung") the cause of which is still unknown.

Carbon black particles and other foreign bodies as well as certain cells (e.g. erythrocytes) are quickly phagocytized by the macrophages of the cerebrospinal fluid. Normally there are no macrophages in the cerebrospinal fluid, but according to *Sörnäs* (1974) these may be differentiated from the leptomeningeal cells or free monocytes in 30 min. These macrophages are able to phagocytize e.g. carbon black within a few minutes after the admixture.

In what way the macrophages leave the subarachnoid space is not known. One explanation could be that they follow the dural sheaths of the spinal nerves (*Armig* 1948). Another explanation

could be that the macrophages penetrate directly through the walls of the cerebrospinal vessels. The predominantly perivascular localization in the brain tissue supports this assumption. However shortly after subarachnoid haemorrhage the cerebrospinal fluid is often clear while perivascular macrophages containing haemodermin may be found many years after a cerebral haemorrhage.

It is not unlikely that macrophages are arrested mechanically by the pulmonary capillaries due to their larger diameter and migrate into the alveolar system thus occurring as carbon black loaded septal cells.

Thus, the elimination of intruding cells or foreign bodies from the cerebrospinal fluid seems to take place at least partly by pulmonary elimination by means of macrophages. The validity of this assumption is now being tested by investigation of expectorate from patients after myelography.

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BILIARY HAMARTOMAS (von MEYENBURG COMPLEXES) IN LIVER NEEDLE BIOPSIES

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Thomsen, N. Biliary hamartomas (von Meyenburg complexes) in liver needle biopsies. *Acta path. microbiol. scand. Sect. A*, 86: 93-99, 1978.

2000 consecutive liver needle biopsies were examined for the occurrence of biliary hamartomas (von Meyenburg complexes, microhamartomas). In 12 biopsies (corresponding to 0.6 per cent) a total of 15 hamartomas were found as 2 biopsies contained 2 and 3 hamartomas, respectively. The incidence, morphology, location and differential diagnosis are discussed. In serial sections a correspondence between all the lesions in each hamartoma was found. In no case the diagnosis of biliary adenoma, compound hepatic fibrosis or polycystic liver could be made. The hamartomas are of no clinical importance.

Key words: Liver needle biopsy, biliary hamartomas, von Meyenburg complexes, microhamartomas.

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Received 24 ix 77 Accepted 24 ix 77

The purpose of this report is to investigate the incidence of biliary hamartomas (von Meyenburg complexes, microhamartomas) in a consecutive material of percutaneous liver needle biopsies. Earlier communications are practically all based on autopsy findings and surgical biopsies.

In addition the author attempts to elucidate the morphology and possible relations to other liver diseases.

MATERIAL AND METHODS

The material consists of 12 percutaneous liver needle biopsies all with biliary hamartomas. They were obtained by examining 2000 consecutive liver needle biopsies received at the Pathological Anatomical Institute of the Hvidovre Hospital from 7 medical departments in

Copenhagen during the period November 1967 to June 1970. The liver biopsies have been performed a *la* Mengesha when there was anamnestic or clinical suspicion of liver disease.

The tissue has been fixed in neutral formalin and embedded in paraffin. The biopsies are 1-1.4 mm thick and 1.0-4.5 cm long. Fifty-two sections were cut on a rotary microtome from each biopsy. In 3 cases a further 70 to 100 serial sections were cut. The sections are approximately 6 µm thick.

A biliary hamartoma is defined according to W. H. O. (15) as a benign lesion consisting of a collection of bile ducts set in a fibrous stroma which is frequently hyalinized.

Initially 58 biopsies were selected for further evaluation. Only 12 biopsies were found to fulfill the diagnostic criteria. The included biopsies contained one or more portal tracts in which bile ducts were slightly ectatic and/or increased in number but without the characteristic connective tissue component.

RESULTS

Biliary hamartomas were found in 12 biopsies, equalizing 0.6 per cent of the examined biopsies. A total of 15 biliary hamartomas were registered as 2 biopsies contained 2 and 3 biliary hamartomas, respectively. There were 3 males and 9 females in the age range 48 to 76 years. The principal histological diagnosis and the final clinical diagnosis are shown in Table I. It appears that one patient was suspected for polycystic disease.

Microscopic Examination

The histological picture only varied a little, a typical example is shown in Fig. 1. All the biliary

hamartomas were located interlobularly in close relation to portal tracts. The connective tissue of the hamartomas always merged directly with the connective tissue of the adjacent portal tract (Fig. 1). The hamartomas measured from 300 to 1100 μ were not encapsulated, but appeared well demarcated. However, in one case (No. 4, Fig. 2) the bile ducts were seen to interdigitate with the liver cell plates.

The epithelium was cubic to low columnar. There was only a slight variation in the height of the epithelium within the single lesion. It was characteristic that the epithelium in the smaller hamartomas was higher compared to the epithelium in the larger lesions.

TABLE I Twelve Cases with Biliary Hamartomas

Case no	Age	Sex	Indication for biopsy	Histological diagnosis	Final clinical diagnosis	Numbers of biliary hamartomas
1	57	♂	Control of earlier biopsy with steatosis	Slight steatosis	Steatosis Chronic alcoholism	1
2	65	♀	Acute hepatitis	Slight non-specific changes, late stage of acute hepatitis?	Acute hepatitis	1
3	58	♂	Atherosclerotic cardiac disease, jaundice	Slight non-specific changes	Myocardial infarction	1
4	68	♂	Cirrhosis?	Slight steatotic, moderate hemosiderosis	Chronic alcoholism	1
5	74	♀	Hepatic disease?	Extramedullary hemopoiesis	Polycystic disease? myelofibrosis	1
6	55	♂	Cirrhosis? Chronic alcoholism	Moderate-severe steatosis	Primary atypical pneumonia - steatosis	1
7	66	♂	Steatosis - cirrhosis?	Cirrhosis	Chronic bronchitis Pulmonary emphysema	1
8	48	♂	Steatosis? Chronic alcoholism	Slight steatosis	Steatosis Chronic alcoholism	3
9	76	♀	Hepatitis?	Slight non specific changes	Hemorrhagic gastritis	1
10	60	♂	Cirrhosis	Slight steatosis	Cirrhosis	1
11	55	♂	Steatosis/cirrhosis? Chronic alcoholism	Slight moderate hemosiderosis	Neurotic depression Chronic alcoholism	1
12	63	♂	Steatosis/cirrhosis	Severe steatosis	Steatosis	2

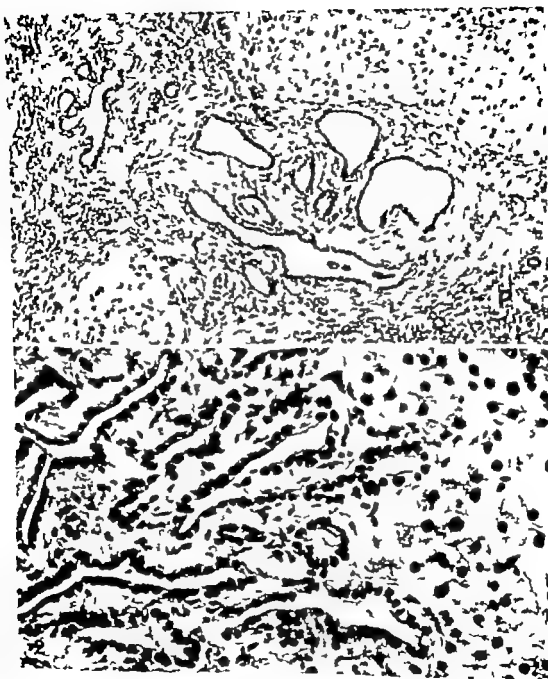


Fig. 1 Typical biliary hamartoma located between 2 portal tracts (P). Note the tubular and cleft-shaped lumina with varying degrees of ectasia. H.E. 100.

Fig. 2 Part of the hamartoma from case no. 4. All the lumina are cleft-shaped without ectasia. The lumina underlie the liver cell plates. H.E. 250.

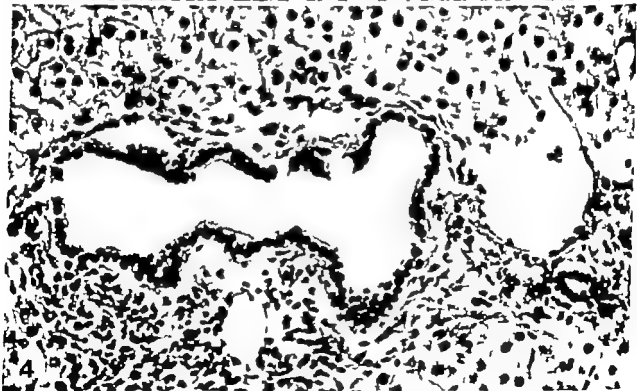
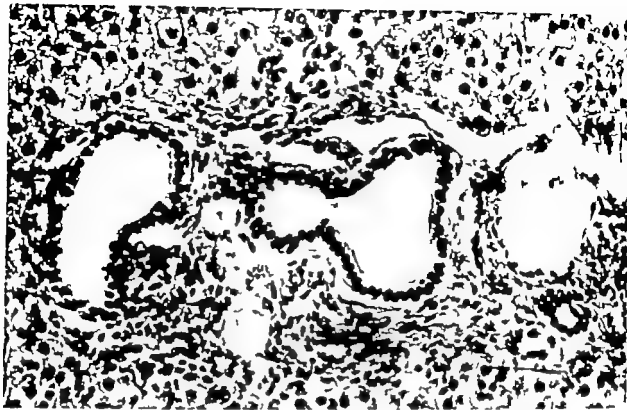


Fig 3 Small hamartoma with 2 lumina Van Gieson $\times 250$

Fig 4 Same hamartoma as shown in *Fig 3* serial section. Only one lumen is seen. Van Gieson $\times 250$



Fig 1 Bilary hamartoma. Two lumina contain bile. H & E $\times 250$

Fig 2 Bilary hamartoma. Note the amorphous and thready cruminate in the lumen. H & E $\times 250$

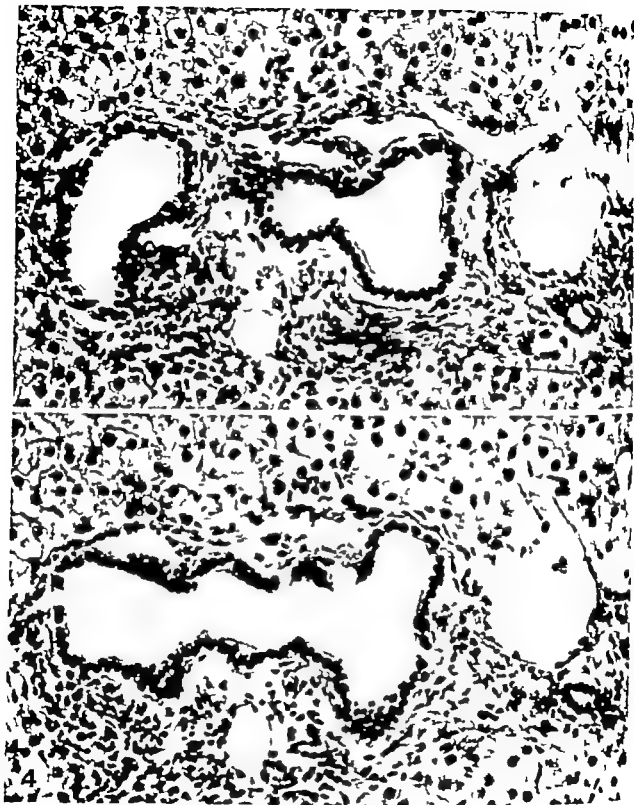


Fig 3 Small hamartoma with 2 lumina. Van Gieson $\times 250$

Fig 4 Same hamartoma as shown in Fig. 3 serial section. Only one lumen is seen. Van Gieson $\times 250$.



Fig. 5 Biliary hamartoma. Two biliary canaliculi in contact. H.E. $\times 250$

Fig. 6 Biliary hamartoma. Note the amorphous and thready exudate in the lumen. H.E. $\times 250$

The lumina were predominantly a little ectatic and ramified. In one section one to twenty lumina could be demonstrated. In serial sections the number of lumina varied. Lumina which in one section were separated were in serial sections seen to communicate (compare Fig. 3 with Fig. 4). By examining serial sections a communication between all lumina was found, i.e. each biliary hamartoma seems to consist of a single ramified lumen.

The shape of the branches was varying. Some were distinctly tubular others were more cleft shaped both types with changing degrees of ectasy. One case (No. 4, Fig. 2) appeared histologically different from the others, because all the lumina were cleft shaped without ectasy. In no case could communication via bile-canalliculi or portal bile ducts be demonstrated. In 5 hamartomas bile pigment in one or more lumina was seen (Fig. 5). In further 6 cases a weak eosinophilic amorphous, granulated or thready exudate in one or more of the lumina was found (Fig. 6). In no case was bile stasis demonstrated in the liver parenchyma.

The bile ducts were situated in an abundant mature connective tissue stroma, which sometimes was partly hyalinized. There were a few small vessels without sclerosing of the walls. The vessels in the adjacent portal tracts were normal. There was no tendency to concentric arrangement around the vessels (Fig. 1). Only a few inflammatory cells were seen in the stroma, most of them lymphocytes, and never more than in the adjacent portal tracts. In two cases the portal tracts showed a slight fibrosis.

The changes in the liver parenchyma appear from Table 1. In no case could the diagnosis of biliary adenoma, congenital hepatic fibrosis or polycystic liver be made.

DISCUSSION

Many different terms have been used to designate biliary hamartomas. Most common are microhamartomas (12) multiple microhamartomas (11) or von Meyenburg complexes (4). Other synonyms are multiple bile-duct hamartomas (1) minute bile duct adenomas (13) cholangioadenomas (3) adenomata (10), multiple adenomas (2), fibroadenomata (10), intracapsular aberrant bile ducts (9) and hepatic hamartomata (5). Biliary hamartoma, the term used by WHO, has been adopted in the present paper as the meaning of this expression best covers the described condition, especially as biliary hamartoma is not a true neoplasm.

Biliary adenoma, cystic liver and congenital hepatic fibrosis are of differential diagnostic importance.

The differentiation from biliary adenoma is

hardly fully elucidated. The definition of immature biliary adenoma of WHO is: no more composed of proliferating small bile ducts lined by normal appearing epithelium set in a loose stroma. Morphological characteristics favouring biliary hamartoma over biliary adenoma is a more abundant and more mature connective tissue, more variation in the size and the shape of the lumina. Furthermore there is often bile in the hamartomas, whereas adenomas never contain bile (2).

The differential diagnosis against cystic liver and congenital hepatic fibrosis is usually easy because biliary hamartomas are focal lesions while the others are diffusely distributed throughout the liver. It should, however, be considered that some also claim the existence of transitional forms (7, 8, 14).

In the presented material biliary hamartomas were found in 12 out of 2000 examined livers corresponding to 0.6 per cent.

It is very probable that all 12 cases represent multiple hamartomas, since the possibility of catching a solitary lesion in a needle biopsy not comprising approximately 1/50 000 of the total liver parenchyma (12), is minimal. This is in accordance with several reports in the literature (1, 7, 8, 9).

To my knowledge there are no publications on the incidence of microhamartomas in liver biopsy materials. Earlier communications deal with autopsy findings and surgical biopsies. Chang (1) found multiple biliary hamartomas in 0.69 per cent of 875 consecutive autopsies. Alferkel (7), over a period of 36 years, demonstrated 70 cases of cystic liver corresponding to an incidence of 0.15 per cent. In 25 of the livers biliary hamartomas were in addition registered. No statement of the incidence of biliary hamartomas in non-cystic liver was given. Thommesen and Christoffersen (14) have in a prospective study of 707 consecutive autopsies found biliary hamartomas in 2.8 per cent. In this investigation all the livers were sliced into sections with an interval of one half cm. Most of these hamartomas were not seen macroscopically. The 2.8 per cent is most likely a better expression of the true incidence than the 0.6 per cent.

In the present material the age distribution for cases with biliary hamartomas does not differ from the age distribution for all the examined biopsies.

The histological picture and the interlobular focalization corresponds to earlier reports (1, 7, 8, 9). Von Meyenburg (8) found communication between all the lumina of the single lesions. More recent authors do not seem to be especially aware of this, but the observation is in agreement with our findings.

Some authors find areas of direct transition from liver cell plates to the epithelium in some biliary hamartomas (1 7 8). This is not seen in this investigation. No communication to bile ducts in the adjacent portal tract is seen. Nevertheless many biliary hamartomas do contain bile pigment which means that there must be some kind of connection to the bile duct system outside the hamartomas. Only Moschowitz (9) did not find bile pigment in his cases. Jørgensen (6) has by three dimensional reconstruction of the bile ducts in infantile polycystic disease and congenital hepatic fibrosis shown a special shape of the bile ducts in the so-called aductal plates arranged concentrically around vessels. As biliary hamartomas are often found in cystic liver one could expect the same configuration in biliary hamartomas. This is not supported by the present investigation.

Most authors consider the lesion to be a hamartoma (1 11 15). This is in accordance with the well known fact that biliary hamartomas are associated with congenital anomalies such as polycystic liver (7) and anomalies in other parts of the body e.g. polycystic kidney (1 9 10).

It is questionable if biliary hamartomas ever undergo malignant transformation. Hamer (4) has described a case with two foci of adenocarcinoma in a liver and suggested that the tumours arose from biliary hamartomas. However this patient had in addition adenocarcinoma of the pancreas. Willis (16) has described adenocarcinoma arising in a cystic liver. In the present material no neoplastic changes were seen.

Large biliary hamartomas may give differential diagnostic problems on naked-eye inspection and can be difficult to distinguish from tumours, especially metastases (3). Clinically biliary hamartomas are without importance (11). This is supported by the varied clinical diagnosis in this material.

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SCANNING ELECTRON MICROSCOPY OF NEOPLASTIC NEUROGENIC RAT CELL LINES IN CULTURE

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Haugen, A. & Lerum, O. D. Scanning electron microscopy of neoplastic neurogenic rat cell lines in culture. *Acta path. microbiol. scand. Sect. A*, 86: 101-110, 1978.

The surface structure of a series of malignant neurogenic rat cell lines in culture has been investigated by scanning electron microscopy. Neoplastic transformation was induced by a single transplacental administration of the carcinogen ethylnitrosourea (ENU) to BD-IX rats on the 18th day of gestation. The malignant cell lines were established either by transplantation into culture of cells from solid gliomas or neurofibrosarcomas developed in the offspring, or by transfer of fetal brain cells to culture where they subsequently underwent malignant transformation. A high degree of surface activity was observed, as evidenced by microvilli, filopodia, ruffling membranes and zonal blebs. Surface activity was highest in cell cultures giving rise to glioma-like tumours upon re-implantation into syngeneic hosts, and low in those giving rise to neurofibrosarcoma-like tumours, with one exception. The lowest surface activity was seen in cell lines which were not tumourigenic. High surface activity was strongly correlated with a high degree of aneuploidy. No correlation was apparent with other properties of the neoplastic cell lines, e.g. stem line ploidy and population doubling time in cell culture.

Key words: Gliomas, neurofibrosarcoma, scanning electron microscopy, cell culture.

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Received 28 ix 77 Accepted 28 ix 77

The use of scanning electron microscopy is a valuable tool for the investigation of surface alterations in malignant cells as compared to their normal counterpart in the tissue of origin. Analyses have been carried out on different mammalian cell lines, including main and different laboratory animals (see Porter *et al.* 1973; Vesely & Boyde 1973; Gonda *et al.* 1976). In this study we have investigated surface properties of neoplastic cell lines in culture derived from the rat central nervous system. Neoplastic transformation occurred after transplacental administration of the alkylating neurotoxic N-ethyl N-nitrosourea (ENU), and investigations were performed both on cells derived from neuroectodermal tumours developed *in vivo*

and from brain cells which had undergone neoplastic transformation *in vitro*. It was of particular interest to see if surface alterations observed by scanning electron microscopy were correlated with other characteristics of neoplastic cells, such as aneuploidy, growth rate in culture, and tumourigenicity.

MATERIAL AND METHODS

Animals and cell lines. Rats of the inbred BD IX-strain (Druckery 1971) were used.

Altogether 12 cell lines were established. Three cell lines (GV1C, NV1C, TV1C) originate from neuroectodermal tumours induced in the offspring by an intravenous pulse of ENU (75 mg/kg body weight) to

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pregnant BD IX rats on the 18th day of gestation. The cell lines were established from explanted tumours as described earlier (Larum & Rajewsky 1975).

The other type of permanent neoplastic cell lines originated from fetal brain cells transferred to monolayer cell culture shortly after exposure to ENU *in vivo* (BT1C - BT9C Larum & Rajewsky 1975) GVIC, NVIC, TVIC and BT1C - BT7C were developed at the Max Planck Institut für Virusforschung Tübingen, Germany (Larum & Rajewsky 1975), and BT8C and BT9C were obtained in this laboratory. All cell lines were tumorigenic by s.c. or transplantation into BDIX-rats, with the exception of BT8C where all attempts have so far been negative (> 20 culture passages).

The cells were cultured on sterile ethanol washed glass coverslips in 60 mm culture dishes (Costar California) in Eagle-Dulbecco's medium (Flow Glasgow) supplemented with a four fold concentration of non-essential amino acids and 10% heat inactivated calf serum, penicillin (50 units/ml) and streptomycin (50 µg/ml) in a humidified air atmosphere with 5% CO₂ at 37°C. All cell lines were regularly tested for mycoplasmas by aerobic and anaerobic incubation in agar medium, and only mycoplasma-free cells were used. When not otherwise specified cells were harvested in late log phase. For each cell line, coverslips from 5-10 replicate experiments were investigated.

Scanning Electron Microscopy (SEM) Cultured cells were fixed for 2 hours at 37°C in 2% glutaraldehyde made up in 0.1M cacodylate buffer at pH 7.3. Coverslips were then washed three times with cacodylate buffer postfixed for one hour at room temperature with 1% OsO₄ made up in the same buffer dehydrated in increasing concentrations of ethanol and critical-point dried using liquid CO₂ (Anderson 1951). Ethanol served as an intermediate fluid. Thereafter the coverslips were mounted on stubs and vacuum-coated with a thin film of gold. Ultrastructural investigation was performed with a Philips SEM 500 microscope at 25 kV.

RESULTS

Cell lines obtained from solid tumours (V lines). By phase contrast microscopy all three lines exhibited a neoplastic growth pattern with a criss-cross arrangement of cells as well as numerous «piled up» foci as earlier described (Larum *et al.* 1977). By SEM the line NVIC was mainly composed of flat, polymorphic cells and few glia-like cells with long cytoplasmic processes. Ridge-like ruffles and few microvilli and zeiotic blebs were present on the cell surface (Table 1 Fig. 1). The cells of the trigeminal neurinoma-derived line TVIC showed flat cells with little surface «activity». Some microvilli and blebs were observed (Fig. 2). However the cells of the mixed glioma-derived line GVIC possessed a very high number of microvilli as well as filopodia,

ruffles and blebs. Three morphological variants were characteristic for the GVIC line, bi-tripolar cells (flat cells and spherical cells (Fig. 3-5)). They tended to occur spontaneously in different uncultured cultures, and could partly be passaged as separate variants. However cellular morphology was often variable from passage to passage. In addition, bi-phase cultures tended to be composed of glia-like bipolar cells, while confluent cultures contained a higher number of round cells which often detached from the glass or plastic surface. In all three variants the same high degree of surface activity was found.

Cell lines obtained by neoplastic transformation *in culture* These cell lines usually grew in several layers with a «criss-cross» pattern and many piled-up foci. By light microscopy the cells were partly glia like with many slender cytoplasmic processes, and partly more flat with shorter processes. Giant cells were common. These features were also observed by SEM but the heterogeneity of the different cell types observed by light microscopy was not so pronounced. Depending on the cell line, filopodia, ruffles, blebs and many microvilli could be observed. A semiquantitative estimate of these features of the cell surface is given in Table 1.

When compared with the histology of solid tumours obtained by re-implantation into BD IX rats, the highest surface activity and especially the highest density of microvilli appeared to be correlated with those cell lines which gave rise to glioma-like tumours (BT1C, BT3C, BT5C and BT9C, Table 1). However BT4C which gave rise to neurinoma-like tumours also exhibited a high surface activity. BT7C, which also grows as a neurinoma-like tumour *in vivo*, exhibited a low surface activity in culture in the same way as NVIC and TVIC (Table 1). The lowest degree of surface activity was observed in the only permanent cell line which so far has not produced tumours *in vivo*, BT8C. Typical pictures of the morphology of the different BT lines are shown in Fig. 6-11.

The highest degree of surface activity with especially numerous microvilli and filopodia was observed in two strongly aneuploid lines, BT3C (near hexaploid), and BT5C (near-tetraploid). However a similar surface structure was also seen in the line GVIC which is near-diploid. Except for the line BT3C, which had the shortest time from implantation into animals until tumour formation (Table 1), there was no correlation between the surface structure and activity on the one hand, and the latency time until tumour formation after re-implantation on the other hand. Similarly there was no obvious correlation between population doubling time in culture and surface activity.

TABLE 1 *Series of the neoplastic Neurogenic Cell Lines*

	Morphology (phase contrast microscopy)	Morphology (SEM)					
		micro- villi	filopod- us	rafting mini- branes	apical blebs	plasty feet-like	tp
GV1A (lateral plexus)	I fusiform, bipolar cells II round forms III flat, bipolar, tripolar cells	+		+		2.0	21
NV1A (peripheral neurons)	polymorphic cells with few cytoplasmic processes					4.0	18
TV1A (trigeminal neurons)	flat, polymorphic cells	()		()	()	2.0	29
Neoplastic neurogenic cell lines transformed in vitro							
BT1C	mainly flat, polymorphic cells with cytoplasmic processes			()		2.0	32
BT2C	flat, polymorphic cells					4.2	30
BT3C	polymorphic globe-like cells with processes, many giant cells					6.4	33
BT4C	polymorphic cells with processes					3.0	21
BT5C	polymorphic cells with processes					3.1	26
BT6C	flat, polymorphic cells					2.6	37
BT7C	flat, polymorphic cells					2.0	30
BT8C	flat, polymorphic cells			+	()	3.0	25
BT9C	mainly fusiform, bipolar cells						

The dominating surface morphology of the cells was evaluated semi-quantitatively: + low number ++ moderate number +++ very high number (see also Figs). Data for plasty (modal G₁-DNA content) population doubling time (tp) and time from reimplantation into baby 1X rats until first touch of palpable tumours (tp) are partly compiled from *Lerner et al* (1977). Histologically NV1A, TV1A, BT4A, and BT7A are neurosphere-like tumours, GV1A is a mixed glioma-like tumour, the other cell lines gave rise to complex glioma-like tumours (in these lines the designation C means culture line, and A transplantable animal tumour, examples NV1C/NV1A).

Fig 1 Surface structure of NV1C cells, originating from a neurosphere of the plexus lobosomalis. Only few microvilli, blebs and occasional ridge-like ruffles are present on the cell surface. Magnification $\times 1600$.

Fig 2 Surface structure of TV1C cells, originating from a Trigeminal neurosphere. The cells are flat, polymorphic, with low surface activity. Some of the clefts between cells may be due to shrinkage during processing. Magnification $\times 1600$.

Fig 3 GV1C cells originating from a mixed glioma. Most morphological variants with bi- and tripolar globe-like cells at confluency. Magnification $\times 1600$.

Fig 4 GV1C cells, morphological variant with round cells, showing many microvilli, and also ruffles and blebs. Magnification $\times 1600$.

Fig 5 GV1C cells with flat morphology by light microscopy. In addition to numerous microvilli, many tightly-extended filopods are seen at the margin of the cells. Blebs are also common. Magnification $\times 3200$.

Fig 6 BT3C cells, which showed the highest number of microvilli on the cell surface. Magnification $\times 7600$.

Fig 7 Surface structure of BT4C cells with moderate numbers of microvilli, ruffles and blebs. Magnification $\times 15200$.

Fig 8 A piled-up focus of BT5C cells with round cells in the middle and more fusiform cells at the edges. A high degree of surface activity is seen. Magnification $\times 800$.

Fig 9 Surface structure of BT6C cells. Most cells have a moderate number of microvilli and are nearly epithelial. Magnification $\times 1600$.

Fig 10 Flat cells of the non-tumourigenic BT9C line. A rather low degree of surface activity is seen, although some blebs and ruffles are present. Magnification $\times 1700$.

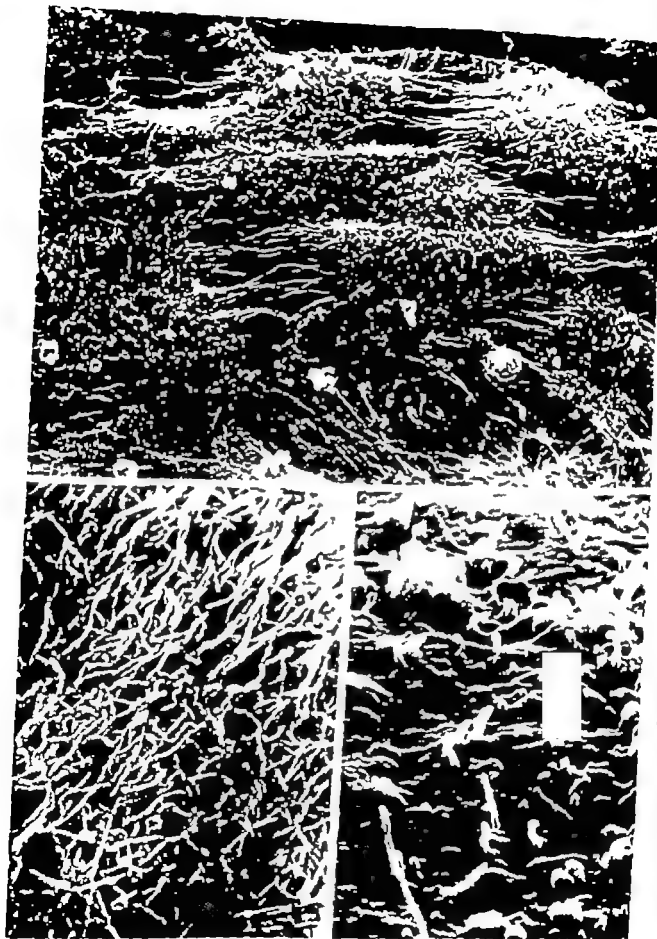
Fig 11 Surface of BT9C cells showing many small ridge-like ruffles and microvilli. Magnification $\times 12800$.



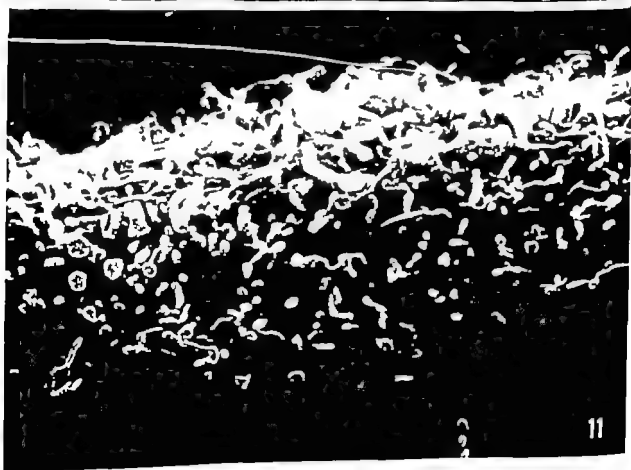












Neoplastic cells in culture often have a characteristic, though not specific, surface micro-structure. A high number of microvilli, filopodia, and ruffling membranes as well as zeiotic blebs have been described in various types of cancer cells (see Porter *et al.* 1973; Melick & Løngrenback 1976; Ambros *et al.* 1975; Gonda *et al.* 1977). These structures, as demonstrated by scanning electron microscopy are expressions of an actively moving, more or less viscous membrane, whose shape may change very rapidly. Thus, Price (1967) could show that zeiotic blebs developed within 8 seconds and retracted within one minute. In addition, surface ruffles which may be connected with the pinocytotic activity of cells may become apparent as early as one hour after viral transformation of fibroblasts in culture (Ambros *et al.* 1975). Culture conditions (Prestbrook *et al.* 1975) as well as population density and cell cycle phase may strongly affect the surface activity as expressed by micro-structure (Fox *et al.* 1971; Rubin and Everhart 1973; Porter *et al.* 1973). The highest number of microvilli is generally found in the G₁-phase, and cell cycle differences may account for some of the heterogeneity observed (see *fax.* Fig. 7 and 9).

The surface microarchitecture of the present malignant neurogenic rat cell lines is in accordance with other observations on human gliomas (Pawels 1975 a & b; Ahnström *et al.* 1976). These authors also noted a strong variability among the different types of cell lines, the deviation from normal being mainly quantitative.

The micro-structure of the present cells is clearly different from that of normal fetal rat brain cells in culture. Part of the latter appear as flat epithelioid cells which have a very low surface activity and may be induced to differentiate to a glia-like morphology by various means, such as extracts from adult brain (Lim & Mizumoto 1974 and Lim *et al.* 1977). Thus we observed no ruffles, some blebs and few microvilli on the cytoplasmic processes in differentiated astrocyte-like cells (Hansen and Løngren 1978).

The present observations are compatible with the assumption that cell lines with a high surface activity give rise to strongly anaplastic tumours, while a lower surface activity is correlated with the production of more differentiated neuroma-like tumours (see Figs.). Similarly strongly aneuploid cell lines may have a higher surface activity than those with near-diploid values. One might therefore wonder whether all these features are connected in terms of tumour progression.

On the other hand, Ahnström (1976) only found a low correlation between the degree of malignancy

and surface activity when studying different benign and malignant human astrocytomas. A similar conclusion was drawn by Pawels (1975 a & b) and Wetzell *et al.* (1977) found only a small difference between neoplastic fibroblast-lines in culture.

It should also be noted that we found no correlation between surface activity and the time from re-implantation until tumour formation in animals (with the exception of the strongly aneuploid line BT3C). Here, however it should not be overlooked that the cell type dominating culture may not be the one with the strongest invasive properties *in vivo*. Thus we have observed that several of these cell lines, when growing as solid tumours, undergo a selection for subpopulations with a near-diploid modal DNA value (Løngren and Mørk 1976; Løngren *et al.* 1978). High surface activity as observed by microvilli, ruffles, or blebs, as well as chromosome aberrations (Sawberg & Sakurai 1974), may thus be a secondary result of and not causally connected with, neoplastic transformation. Further research into earlier stages of carcinogenesis and the appearance of characteristic changes in the surface microstructure of preneoplastic cells may elucidate what changes are of primary importance for the process of malignant transformation.

This investigation was supported by the Norwegian Cancer Society. We thank Mrs Eivabekken and Miss Gro Odland for expert technical assistance. We are also greatly indebted to Dr Øivind Ake for testing the cultures for Mycoplasmas. The ultrastructural investigation was performed at the Laboratory for Clinical Electron Microscopy and we thank Professor H. Dales for valuable advice and discussion during this work.

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EFFECT OF LONG TERM ADMINISTRATION OF VARIOUS ALCOHOLIC BEVERAGES ON THE IN VITRO INCORPORATION OF ^3H LEUCINE INTO PROTEINS IN RAT CEREBRAL CORTEX CEREBELLUM AND LIVER

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Jarlstedt, J., Jordö, L. & Olsson, R. Effect of long-term administration of various alcoholic beverages on the *in vitro* incorporation of ^3H leucine into proteins in rat cerebral cortex, cerebellum and liver. *Acta path. microbiol. scand. Sect. A*, 86: 111-116, 1978.

The incorporation of [^3H] - leucine into protein from anterior and posterior cerebellum, cerebral cortex and liver was studied in rats given 50% of calories as ethanol, brandy, whisky, gin, red wine or isocaloric amounts of glucose together with diets with moderate or low protein-vitamin content for 8-9 months. Higher incorporation rates were usually observed with higher protein-vitamin administration. Red wine and brandy rats usually had the highest, ethanol and gin rats usually the lowest incorporation rates. The incorporation rate thus increased with amount of congeners present.

Key words: Alcoholic beverages, amino acid incorporation, cerebellum, cerebrum, liver, rat, malnutrition.

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Accepted 23.11.77

It has been estimated that alcohol often provides about half the caloric requirements in a chronic alcoholic (19). This obviously creates a risk of malnutrition with deficient intake of all important food constituents, notably proteins and vitamins.

The combined effects of ethanol and a poor diet may in the long run induce degenerative changes in the central nervous system, resulting in, for instance, atrophy of the cerebral cortex and cerebellar degeneration (1, 7, 18).

Apart from ethanol, the consumed alcoholic beverages contain small and variable amounts of other substances known as congeners. These are methanol, furfural, fusel alcohols, etc. (13, 19). The possible adverse effects on nervous system functions of these congeners have long been disputed.

Most investigators tend to regard ethanol *per se* as the only harmful factor in alcoholic beverages (for review see *Wattgren and Barry* 19), while results presented by other authors suggest that the toxicity varies between different spirits (4, 9).

As far as we know, there has been no previous study of protein synthesis in the nervous system where rats have been fed diets containing various alcoholic beverages for a prolonged period. In the present study rats were fed 50% of the total calories as ethanol, brandy, gin, whisky or red wine, together with either a protein and vitamin sufficient or a low protein, low vitamin diet for 8-9 months. The incorporation rate of radioactive leucine into cerebral and cerebellar proteins was measured *in vivo*. Separate investigations were performed on anterior and posterior parts of the cerebellum, since

previous studies have demonstrated differences in sensitivity to ethanol between these two regions (16). The incorporation into liver proteins was also determined in order to provide an evaluation of whether possible changes were brain-specific or not.

MATERIAL AND METHODS

Male Sprague Dawley rats, weighing 175–183 g at the start of the experiments were used for the study. Five animals were housed in every cage.

Diets The rats were given semi-liquid diets as the sole source of food and water. Two different diets were used in which the calories derived from fat, protein, carbohydrate and ethanol were as follows (in % of total calories)

	Diet A	Diet B
Fat	6	20
Protein	14	6.5
Carbohydrate	30	23.5
Ethanol	50	50

Details of the composition and preparation of the diets have been published earlier (10).

Alcohol was isocalorically given as ethanol, whisky, brandy, gin and red wine of cheap qualities. A control

group received isocaloric amounts of glucose instead of alcohol. Each subgroup initially consisted of 10 animals.

After 8–9 months the animals were killed by exsanguination through aortic puncture under ether anaesthesia. During 24 hours before death the alcohol groups were reared on the diet of the glucose-control group. The rats in the present series were also subject to other biochemical and morphological studies that have been presented elsewhere (10, 11, 12). Because of its time-consuming analyses which had to be performed immediately *post mortem* the period of sacrifice had to be extended to one month. The sacrifice of the animals of each subgroup was evenly distributed over this period. The serum ethanol concentration was determined in 15 animals from different subgroups after 8 months of 50% alcohol diets by aid of gas chromatography. Tail vein punctures were performed at different times of the day. At sacrifice, the brain was rapidly removed. Pieces of cerebral cortex, anterior and posterior cerebellum and liver were rapidly removed, sliced with a MacIlwain tissue chopper set at 0.4 mm and transferred directly into the incubation vessels. The vessels were gassed for 60 sec with 100% oxygen. The incubation medium contained: 35 mM Tris HCl, pH 7.6; 5 mM Na^+ -phosphate buffer, pH 7.6; 5 mM KCl; 120 mM NaCl; 2.5 mM MgCl_2 ; 5 mM glucose; 2.5 mM ATP and H^3 -leucine 40 $\mu\text{Ci}/\text{ml}$ medium (4.5- H^3 leucine, spec. act. 22 Ci/mM). The Radiochemical Centre, Amersham. All media were freshly prepared and millipore (0.22 μ) filtered. The slices were incubated for 30 minutes, and the incubation was stopped by transferring the incubation vessels to ice. The medium was filtered off, and the

TABLE 1 Summary of Statistically Significant Differences in H^3 -leucine Incorporation between Different Alcoholgroups in Different Investigated Tissues

	Number of animals	Ant. cerebellum	Post. cerebellum	Cerebral Cortex	Liver
E	A 5 B 5				
B	A 5 B 9	E G	E G	E	E
W	A 7 B 6	G	G		E
G	A 3 B 5				
RW	A 5 B 5	E GC, G, W, B, E	E GC, G	E	
GC	A 5 B 8	E	E G	E	E

From this table it is evident that, for example, brandy B rats had significantly higher incorporation into protein in anterior and posterior cerebellum than gin rats given the B diet, and higher incorporation into liver protein than ethanol rats given the B diet. For explanation of abbreviations, see text of Fig. 1.

liver samples were homogenized in excess volumes (5 ml/100 mg tissue) ice-cold 5% TCA. After centrifugation of the homogenates, aliquots were taken from the TCA supernatant for determination of TCA soluble activity and calculated as CPM per mg protein in the homogenate. The TCA insoluble material was washed three times with 5% TCA, the last time after heating the samples in boiling waterbath for 20 min. After cooling on ice the pellet obtained after centrifugation was lipid extracted with ether-absolute ethanol (1/1) and subsequently with pure ether. The lipid-extracted material was centrifuged, and the protein content in the pellet was determined according to Lowry *et al.* (14). The radioactivity was determined by liquid scintillation counting and expressed as CPM per mg protein. Counting efficiency was 26% and the mean background activity was 19 CPM.

Studies using identical labelling techniques have shown that the *in vitro* incorporation of 35 S-leucine into

brain protein is linear with time from 10–60 min (3, 5). Haglid and Hansberger (5) showed that the *in vitro* incorporation rate of 35 S-leucine into brain protein is not changed by adding 1, 3 or 5 times the plasma level of a standard amino acid mixture to the above-mentioned incubation medium.

Fisher's non-parametric permutation test was used for the statistical comparisons. The level of significance was 5%.

RESULTS

General Remarks

The mean daily consumption initially was about 380 kcal/kg and decreased to about 160 kcal/kg towards the end of the experiments. The mean daily alcohol consumption over the whole period was about 20 g/kg. Alcohol rats except red wine rats in

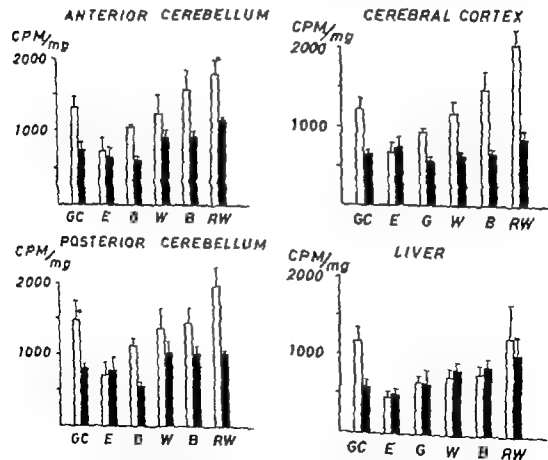
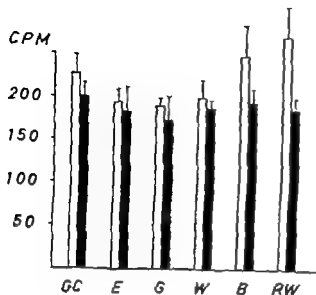
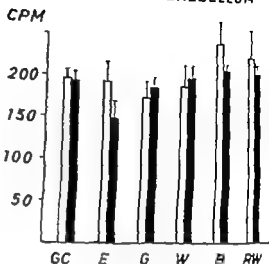


Fig. 1 35 S-leucine incorporation (CPM per mg protein) into protein from anterior cerebellum, posterior cerebellum, cerebral cortex and liver in rats fed 50% of calories as ethanol (E), whisky (W), brandy (B), gin (G) or red wine (RW) together with diets with moderate (A - white) or low (B - black) protein and vitamin content. In glucose control groups (GC) the alcohol calories were replaced by glucose. Asterisks indicate statistically significant difference between A and B groups. A summary of statistical differences between groups given the same diet but different alcoholic beverages or

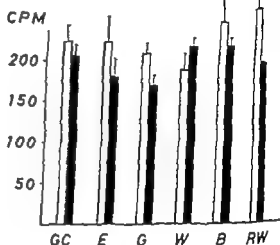
CEREBRAL CORTEX



ANTERIOR CEREBELLUM



POSTERIOR CEREBELLUM



LIVER

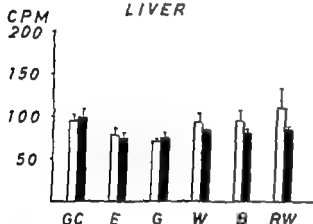


Fig 2 TCA soluble radioactivity in homogenates (CPM per mg total proteins in the homogenate) from anterior cerebellum posterior cerebellum cerebral cortex and liver in the same groups as in Fig. 1 The following significant differences were observed

Anterior cerebellum	Brandy B>Ethanol B
	Red Wine B>Ethanol B
Posterior cerebellum	Brandy B>Gm B
	Whisky B>Gm B
Liver	Whisky A>Gm A

No statistically significant differences were detected between groups given the same alcoholic beverage but different diets

series A displayed a slightly higher body weight increase in relation to caloric intake than the alcohol rats in series B whereas no such difference was noticed between the control groups. The serum ethanol concentration, measured in 15 animals at different times of the day during the last two months, varied between 55 and 270 mg/100 ml (mean 143 mg/100 ml) Details on growth of animals, food consumption, body and organ weights, etc. are given in another paper (10).

Biochemical Findings

With the exception of the ethanol group the rats given the moderate protein diet (A) usually had higher incorporation rates into cerebellar and cerebral proteins than the rats given the low protein diet (B) (Fig 1 and Table 1) This trend was not apparent when studying liver proteins.

The different alcoholic beverages exerted different effects on the incorporation rate, but the pattern of influence was usually the same in the different

James studied. Thus, ethanol and gin rats usually showed the lowest incorporation rates, whereas red wine rats showed the highest. In general, the TCA-soluble radioactivity (reflecting the amino acid pool available for protein synthesis) was somewhat lower in nervous tissue from B rats than from A rats (Fig. 1). Significant differences between different groups were rare, but the same pattern of influence as in the incorporation studies was discernible.

DISCUSSION

The results obtained in the control rats show that the administration of a diet rich in fat and low in protein causes a decreased incorporation of leucine into proteins in brain tissue. Since the uptake of the labelled amino acid (measured as TCA-soluble activity) was not affected, the decreased incorporation is due to a decreased capacity for protein synthesis. Similar results have been obtained in other experiments (8). A tentative explanation of these findings is that the low protein supply caused an adaptation to a lower protein synthesis.

The depressive effect of ethanol on amino acid incorporation into liver proteins, observed in the present study, is in agreement with previous observations. Thus, whereas acute ethanol intoxication (0.45 g/kg, Ashworth *et al.* (2); 8 g/kg, Rubin *et al.* (17)) does not decrease the incorporation of radioactive amino acids into liver proteins, prolonged administration (36% of calories for 24 days) causes a 25% reduction (16). It seems likely that the decreased incorporation found in the tissue from ethanol-treated A rats is due to an impaired protein synthesis, since ethanol did not significantly influence the uptake of the precursor.

Compared to ethanol, brandy and red wine increased the amount of incorporated activity in all investigated brain areas. In fact, the rats given red wine sometimes showed higher incorporation than the controls. The higher amino acid incorporation into CNS protein could be related to an increased uptake of labelled acid reflected in a suggested but only exceptionally statistically significant higher TCA-soluble activity in these groups.

A striking finding was the consistent pattern of beverage-related differences in leucine incorporation in all the tissues studied. This observation is of special interest when considering that the amount of congeners present in the different beverages increases in the same order, i.e. ethanol - gin - whisky - brandy - red wine (13) as the leucine incorporation in the present series (Fig. 1). It is thus obvious that a high amount of congeners does not impair but rather increases the over-all capacity for amino acid uptake and incorporation into protein in the tissues

studied. Unfortunately, rather little is known about the functional importance of such changes in cerebral protein metabolism as those observed in the present study. However, it is noticeable that the differences in effects between the different alcoholic beverages observed in brain tissue were clearly not specific for this tissue, since the same pattern of influence was observed in liver tissue. It is also noticeable that in a biochemical and histological study of the livers in the present series of animals, red wine and whisky appeared to have more deleterious influences on the liver than ethanol or gin (10).

This work was supported with grants from the Swedish Medical Research Council.

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THE MYOCARDIAL CAPILLARY VASCULATURE IN REPEATED PHYSICAL EXERCISE

An Experimental Investigation in the Rat

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Carlsson, S., Ljungqvist, A., Tornöling, B. & Uнге, G. The myocardial capillary vasculature in repeated physical exercise. *Acta path. microbiol. scand. Sect. A* 86: 117-119, 1978.

The neoformation of myocardial blood capillaries was investigated on cardiac autoradiograms from ^3H -thymidine injected rats that had been swimming-exercised for two weeks, rats that had been resting for three months following the exercise, and rats in which the rest had been followed by a second two-week period of exercise. The initial period of exercise induced a significant capillary neoformation in the hearts. The newly-formed vasculature was found of functional significance still at the end of the resting period and available for the increased demands on the myocardial vasculature when the exercise was resumed.

Key words: Myocardial capillary vasculature, physical exercise, rat.

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Received 10 x 77 Accepted 10 x 77

In a series of previous studies we have demonstrated the neoformation of myocardial blood capillaries in hypertrophied hearts of swimming-exercised rats (Ljungqvist & Uнге 1972 and 1973, Ljungqvist *et al.* 1976, Mandache *et al.* 1972 and 1973). The present investigation was undertaken to elucidate whether these newly-formed capillaries undergo involution when the exercise is discontinued for a prolonged period or will remain patent and be available if the exercise is resumed.

MATERIAL AND METHODS

Forty-two female Sprague-Dawley rats with an initial weight of 225 g were used in the experiments. The rats were housed in cages, 4 in each, and were given a standard laboratory diet and tap water *ad libitum*. The exercise consisted in swimming 1 hour each day, 6 days per week, as previously described (Ljungqvist & Uнге 1972).

The experimental groups (I-III) originally consisted of 10 rats each and the control group (IV) of 12 rats. During the experiment some of the rats had to be

eliminated owing to technical failures such as unsuccessful thymidine injections (see below). The final grouping of the rats was the following:

Group I: Nine rats which were exercised for 2 weeks, immediately after which they received a single dose of 2 μCi ^3H -thymidine/g body weight (specific activity 50 Ci/mmol, Amersham, England) intraperitoneally. The rats were killed 24 hours later.

Group II: Eight rats were exercised for 2 weeks followed by a resting period of 3 months and the above injection of labelled thymidine at the end of that period. The rats were killed 24 hours after the injection.

Group III: Ten rats which were exercised for 2 weeks followed by a resting period of 3 months. After this period exercise was resumed for 2 more weeks followed by an injection of labelled thymidine. The rats were killed 24 hours later.

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index of group II significantly lower ($p < 0.01$). This is graphically presented in Fig. 1. The differences between groups I and II and II and III are also statistically significant ($p < 0.01$).

DISCUSSION

The results of the present investigation have confirmed our previous observations of cardiac hypertrophy and increased incorporation of ^3H -thymidine into the cells of the myocardial capillary walls of swimming-exercised rats (Ljungerius & Unger 1973, Ljungerius *et al.* 1976). The latter feature is indicative of an increased DNA-synthesis of the capillary wall cells (Clever 1967) which, in the experimental model used, were shown to be proliferating endothelial cells and pericytes forming new myocardial blood capillaries (Mandeloch *et al.* 1972 and 1973). The degree of ^3H -thymidine incorporation into the myocardial capillary wall cells could therefore be taken as a measure of the intensity in capillary neoformation in the hearts.

That exercise can lead to neoformation of myocardial blood vessels has been shown in previous experiments by other workers in which the capillary density or capillary/muscle fiber ratio has been calculated. Using the former method in exercised rats, Petren and Sjöström (1937) demonstrated that the newly-formed myocardial capillaries were still present after a resting period of two months but they had disappeared after a further month of rest. In recent investigations Leon and Bloor (1968, 1976) made a similar observation on rats that had been exercising for 10 weeks. In these rats newly formed capillaries were still present after a resting period of 6 weeks, but they had all disappeared 4 weeks later.

In the present investigation the interruption of the exercise for three months was found to result in a decrease in thymidine-incorporation into the myocardial capillary wall cells below control levels. This indicates that the stimulus for a capillary neoformation in these animals was even less than in acclimatized animals of the same age, suggesting that the capillaries formed during the exercise were still of functional significance after three months of rest. This period of persistence of newly formed capillaries is far longer than that found by Petren and Sjöström (1937) and Leon and Bloor (1976) but this can be due to differences in mode and intensity of exercise and in methods of investigation.

The fact that capillaries formed during exercise were still available after three months of rest is further supported by the finding that if the exercise was resumed after the resting period capillary neoformation was again induced but to a lesser degree than during the first period of exercise. The absence of cardiac hypertrophy following the second period of exercise remains to be explained and requires further investigations.

This study was supported by the Swedish Medical Research Council (Project no 875-12X 716) and the Research Funds of the Karolinska Institute.

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Group IV Twelve control rats. The group consists of a) 5 rats, in age corresponding to the rats of Group I and b) 7 rats, in age corresponding to the rats of Group II and III. The rats were killed 24 hours after an injection of labelled thymidine.

All rats were killed by an overdosage of ether and the heart/body weight ratios were calculated. The hearts were fixed in 10 per cent neutral formalin and embedded in paraffin. From the paraffin blocks 4 μ thick transverse sections were taken from the ventricle portion of the heart. The sections were placed on glass slides and covered with Kodak AR 10 film for autoradiography according to the stripping film technique. The exposure time was 8 weeks at +4° C, after which the autoradiograms were developed in Kodak D19B and fixed in Kodak acid fixer. The sections were stained through the film with haematoxylin-eosin.

Identification of capillary wall cells and calculation of labelling indices were done on the autoradiograms as described earlier (Ljungqvist & Unge 1973).

Statistical evaluation of differences between the groups of rats was done by analysis of variance (Finner 1962). In order to achieve homogeneity of variance between the groups the analysis was done on logarithmic values of labelling indices.

RESULTS

Body weight, heart weight and heart/body weight ratios of the various groups of rats are shown in Table 1. Although the variations in terminal body weights of the rat groups make an evaluation of their heart/body weight ratios difficult, the previously demonstrated feature of an increased ratio, i.e. cardiac hypertrophy during swimming exercise

TABLE 1 Body Weight, Heart Weight and Heart/Body Weight Ratios ($\times 1000$) in Rats Subjected to Swimming Exercise during 2 Weeks (Group I), in Rats Subjected to Swimming Exercise during 2 Weeks Followed by a Resting Period of 3 Months (Group II) and in Rats Subjected to the Same Treatment as Group II Followed by a Second Period of Swimming Exercise during 2 Weeks (Group III). Group IVa is Composed of Normal Controls Corresponding in Age to Group I and Group IVb of Normal Controls Corresponding in Age to Groups II and III

Group	No of rats	Body weight (g)	Heart weight (g)	Ratio $\times 1000$
I	9	225 \pm 3	0.81 \pm 0.06	3.59 \pm 0.24
II	8	251 \pm 17	0.90 \pm 0.07	3.58 \pm 0.24
III	10	295 \pm 13	0.98 \pm 0.08	3.33 \pm 0.20
IVa	5	274 \pm 4	0.75 \pm 0.02	3.36 \pm 0.18
IVb	7	299 \pm 12	1.06 \pm 0.07	3.56 \pm 0.21

TABLE 2 Labelling Indices of Myocardial Capillary Wall Cells after ^3H Thymidine Injections into Rats Subjected to Swimming Exercise during 2 Weeks (Group I), in Rats Subjected to Swimming Exercise during 2 Weeks Followed by a Resting Period of 3 Months (Group II), and in Rats Subjected to the Same Treatment as Group II Followed by a Second Period of Swimming Exercise during 2 Weeks (Group III). Group IVa is Composed of Normal Controls Corresponding in Age to Group I and Group IVb of Normal Controls Corresponding in Age to Groups II and III

Group	No of rats	Index
I	9	97.56 \pm 39.82
II	8	21.00 \pm 6.04
III	10	63.70 \pm 14.86
IVa	5	45.20 \pm 10.23
IVb	7	42.42 \pm 6.82
IV tot	12	43.58 \pm 8.09

could again be noted (Ljungqvist & Unge 1973). It appears, however, that the ratio subsides during a subsequent 3 months period of rest, and that a second period of exercise does not produce an reappearance of the cardiac hypertrophy, the heart weight rather dropping to subnormal levels.

In Table 2 the labelling indices of myocardial capillary wall cells in the various groups of rats are presented. The two control groups (IVa and b) do not differ in labelling index whereas the indices of groups I and III are significantly higher than the corresponding control values ($P < 0.01$) and the

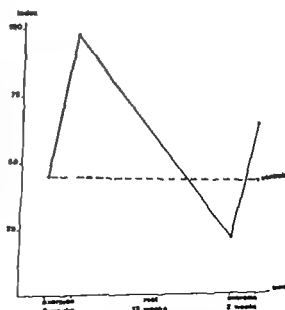


Fig. 1 Labelling indices of myocardial capillary wall cells after ^3H -thymidine injections into rats subjected to swimming exercise - rest - exercise.

THE ISOZYME PATTERN OF CYCLIC AMP DEPENDENT PROTEIN KINASE AND THE DISTRIBUTION OF A CERVICOVAGINAL ANTIGEN IN EXPERIMENTAL CARCINOMA OF THE CERVIX UTERI OF MICE

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Døskeland, S. O., Kalland, T., Stray Breistein, L., Fossberg, T. M., Ueland, P. M. The isozyme pattern of cyclic AMP dependent protein kinase and the distribution of a cervicovaginal antigen in experimental carcinomas of the cervix uteri of mice. *Acta Path. Microbiol. Scand. Sect. A*, 86: 121-130, 1978.

Tissue-sections from 12 methylcholanthrene-induced carcinomas of the cervix uteri of mice were tested for the presence of an antigen normally confined to the cervicovaginal epithelium. The antigen was detected in 10 of the 12 tumours investigated with indirect immunofluorescence, and in all 7 tumours studied with the more sensitive method of mixed haemagglutination. The concentration of the antigen was generally higher in the well-differentiated areas of the tumours, but it was also found associated with solitary tumour cells, apparently invading the stroma. The presence of CVA in the tumours suggests an origin of the tumour cells from the cervicovaginal epithelium. The cyclic AMP dependent protein kinase (EC 2.7.1.37) in the tumour cytosols was studied by chromatography on agarose and DEAE-cellulose. The enzyme showed the same properties as that from normal vaginal epithelium. The tumour cells thus contain an apparently normal complement of this enzyme, which is believed to be responsible for most of the intracellular actions of cyclic AMP.

Key words: Cervixcarcinoma, methylcholanthrene, enzyme, immunological marker, protein kinase, cyclic AMP, chromosomes.

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Received 14 vii 77 Accepted 10 x 77

The effect of prolactin on the incidence of 3-methylcholanthrene-induced carcinomas of the uterine cervix in mice (18) and on some growth parameters of such cells *in vitro* (17) have been reported from this laboratory. Current studies of the effect of dibutyryl cyclic AMP on such cells prompted us to investigate whether the cyclic AMP dependent protein kinase(s) in the tumours differed from the enzyme found in normal vaginal epithelium from the same strain of mice (10). Cyclic AMP (25, 26), as well as its butyrylated analogue (21, 22), are believed to influence the cell by activation of the

intracellular protein kinase. Prolactin has been shown to affect the intracellular concentration of protein kinases in mammary gland epithelium (29). It was therefore decided to study tumours from both neonatally untreated animals and neonatally estrogenized animals, as the latter have an elevated level of circulating endogenous prolactin (31).

An antigenic substance (cervicovaginal antigen = CVA) accumulates in the vaginal tract in response to prolactin (23), as well as in explants of neonatal uterine cervix exposed to dibutyryl cyclic AMP (27). Since the concentration of CVA is governed by factors (cyclic AMP, prolactin) which

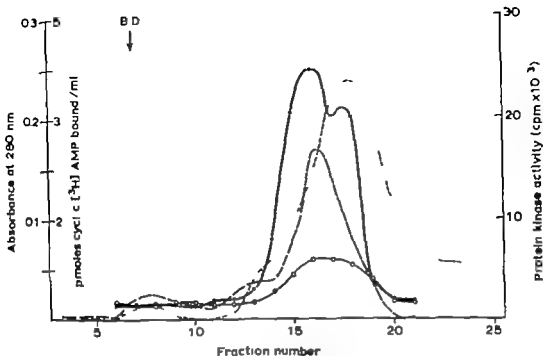


Fig. 1 Sepharose 4B chromatography of carcinoma cytosol. A column (16 x 65 cm) of Sepharose 4B was equilibrated with 10 mM Tris-HCl pH 7.5 containing 4 mM EDTA and 6 mM 2-mercaptoethanol, and 5 ml of cytosol applied. Fractions of 5 ml were collected. Cyclic [3 H] AMP binding (—●—) was measured in 300 μ l aliquots taken directly from the tubes, and protein kinase activity determined in 100 μ l aliquots of fractions detailed as described (9), in the presence of 2 μ M cyclic AMP (●—●) and in the absence of cyclic nucleotide (O—O). The absorbance at 280 nm (---) was measured in a Zeiss PM QII spectrophotometer. The elution of Dextran Blue (Pharmacia) is indicated by the vertical arrow.

tumour cytosols from non-estrogenized animals. The reaction rate was linear with respect to time and concentration of cytosol protein under the experimental conditions used. The proportionality between reaction rate and concentration of cytosol protein ensured that variable tissue levels of the heat-stable inhibitor protein did not affect the kinase activity (9). The kinase was stimulated about 4-fold in the presence of 2 μ M cyclic AMP. The activity (in the presence of cyclic AMP) ranged from 210 to 280 units/mg cytosol protein. There was no indication that neonatal estrogenization influenced either the level or cyclic AMP dependence of cytosol protein kinase. The mean kinase activity per mg cytosol protein was about 20% lower than in pooled non-malignant vaginal epithelium (10), but higher than in vaginal stroma (Ueland & Denkeland unpublished observations).

Gel Chromatography and Ion-Exchange Chromatography of Cytosol

Samples from each of the four tumour cytosols referred to above, were subjected to agarose chromatography. The peak of cyclic AMP binding

activity and the main peak of kinase activity eluted (Fig. 1) like the corresponding activities from normal vaginal epithelium (10). A shoulder of kinase activity was eluted after the main peak (Fig. 1).

The peak fractions from the agarose column (Fr 14–19) were pooled and chromatographed on DEAE-cellulose. Most of the protein kinase activity was eluted between 150 mM and 220 mM KCl and was associated with cyclic AMP binding activity (Fig. 2).

Another peak of cyclic AMP binding activity devoid of kinase activity was eluted at about 80 mM KCl. The material corresponding to this peak sedimented at about 4.5 S upon sucrose density gradient centrifugation (data not shown). The elution profiles shown in Fig. 1 and Fig. 2 are representative for all the four tumours studied, whether taken from estrogenized animals or not.

Histological Examination of the Tumours

All the tumours investigated were confirmed to be frank carcinomas by routine histology. On the basis of the biopsies, nine tumours were classified as well-

may also affect the protein kinase, it was considered of interest also to study the distribution and concentration of CVA in the tumours. In the normal mouse, CVA is confined exclusively to the cervicovaginal epithelium. Its possible occurrence in the carcinomas might thus give clues to the histogenesis of the carcinomas.

MATERIALS AND METHODS

Treatment of Experimental Animals

The mice belonged to a closed, randomly-bred NMRI strain. They were fed a standard pellet diet and given water *ad libitum*. Some of the animals were given subcutaneous injections of 5 µg estradiol-17β (Sigma Chemical Co) in 25 µl olive oil on each of the first five days of life; others were given olive oil only. At the age of 6–9 weeks the animals were laparotomized, and a cotton thread impregnated with a mixture of 3-methylcholanthrene (Sigma Chemical Co) and beeswax (1:5 w/w) was inserted into the uterine cervix (15). The mice that developed palpable tumours were used for experiments (about 2½–3½ months after the threads had been inserted).

Routine Histological Examination

Several specimens were taken from each tumour used in the study fixed in Bouin's solution, embedded in paraffin, sectioned at 6 µm, and stained in hematoxylin and eosin for histological study.

Preparation of Tissue Sections Procedure for Immunofluorescence and Mixed Hemagglutination on Tissue Sections

Three specimens were rapidly removed from different parts of the tumour while the animal was in ether anaesthesia, quickly frozen in a jet of CO₂ and sectioned transversely in a Dittus cryostat kept at about -20° C. The method of indirect immunofluorescence was as described by Forsberg & Kihlstrand (16). Briefly the sections were air-dried at 56° C, immersed in acetone, dried again, and incubated with anti CVA serum for 70 min at 37° C. The sections (after rinsing in phosphate buffered saline pH 7.4) were then incubated for 15 min at 22° C with FITC-conjugated anti-rabbit γ-globulin serum (from goat (Behringwerke AG). The fluorescence was excited by transmitted ultraviolet light (from a high pressure mercury lamp). Preparation of the anti-serum against (partially purified) CVA was as described earlier (13). The indirect mixed hemagglutination technique for the demonstration of antigen in tissue sections was based on the method of Tander *et al* (34). The procedure was modified (13) to allow a relative quantitation of the CVA-concentrations in the sections. The highest dilution of the anti CVA serum giving a density of the indicator corpuscles corresponding to a + (+) reaction on a scale from 0 to +++ is defined as the titer of CVA. This is a measure of the CVA-concentration. Two tumours from estrogenized animals and three from non-estrogenized animals were only studied by immunofluorescence.

Four tumours from neonatally estrogenized animals and 3 tumours from non-estrogenized animals were examined by both immunofluorescence and mixed hemagglutination. The CVA concentrations given refer to the groups of carcinoma cells with the highest titer of CVA in any particular tumour.

Assays for Protein Kinase Activity and Binding of Cytosol [32P]ATP

were as described earlier (9). The cyclic [8-³⁵S]ATP (2 Ci/mmol) and [γ-³²P]ATP were from The Radiochemical Centre, Amersham. The substrate for the protein kinase was whole-calf thymus histone (Type II, Sigma Chem. Co). One unit of protein kinase activity is defined as the amount of activity transferring 1 pool of phosphate into histone per min under the experimental conditions described above.

Preparation of Cytosol from Cervix Carcinomas

The tumour was quickly removed from the ether anaesthetized animal after biopsies had been taken for routine histological examination. It was then grasped in large metal forceps cooled in liquid N₂ and plunged into the nitrogen. After 1–5 weeks in liquid N₂ the tumours were thawed in homogenization buffer. Homogenization and further preparation of cytosol was as described earlier (10).

Gel Chromatography Ion Exchange Chromatography and Sucrose Density Gradient Centrifugation of Cytosol

The procedures for gel chromatography using Sepharose 4B (Pharmacia, Uppsala, Sweden) and ion-exchange chromatography using DEAE-cellulose (DE-52, Rheimann Biochemicals Ltd, Midsand, Neth. U.K.) as well as sucrose density gradient centrifugation (5–20% w/v linear gradient spun for 20 hours at 40 000 rev/min) have been described previously (10).

Karyological Examination

Affected biopsies from carcinomas of the cervix were incubated for 2 hours at 37° C in tissue culture medium (Parker 199 Statens Bakteriologiska Laboratorium, Stockholm, Sweden) containing 0.05 µg/ml of colcemid. The tissue specimens were next placed in a hypotonic solution (75 mM KCl) for 10 min at 22° C, followed by methanol/acetic acid (3/1 v/v) for 2 hours and 60% (v/v) glacial acetic acid for another 2 hours. Droplets from the resulting cell suspension were placed on pre-warmed (50° C) slides, air-dried and the preparation stained with Giemsa solution as described by Buckland *et al* (3). Three tumours two of which were from neonatally estrogenized animals, were studied. Between 20 and 30 metaphases were counted for each tumour.

RESULTS

Characterization of the Protein Kinase Activity in Tumour Cytosol

The protein kinase activity was measured in de-salted samples from two cytosols prepared from tumours of neonatally estrogenized mice and two

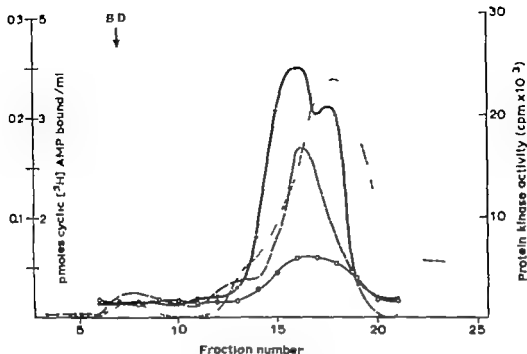


Fig. 1 Sepharose 4B chromatography of carcinoma cytosol. A column (116 x 65 cm) of Sepharose 4B was equilibrated with 10 mM Tris-HCl pH 7.5 containing 4 mM EDTA and 6 mM 2-mercaptoethanol, and 5 ml of tyrosol applied. Fractions of 5 ml were collected. Cyclic [3 H] AMP binding (—●—) was measured in 300 μ l aliquots taken directly from the tubes, and protein kinase activity determined in 100 μ l aliquots of fractions desalted as described (9), in the presence of 2 μ M cyclic AMP (●—●) and in the absence of cyclic nucleotide (○—○). The absorbance at 280 nm (---) was measured in a Zeiss PM Q11 spectrophotometer. The elution of Dextran Blue (Pharmacia) is indicated by the vertical arrow.

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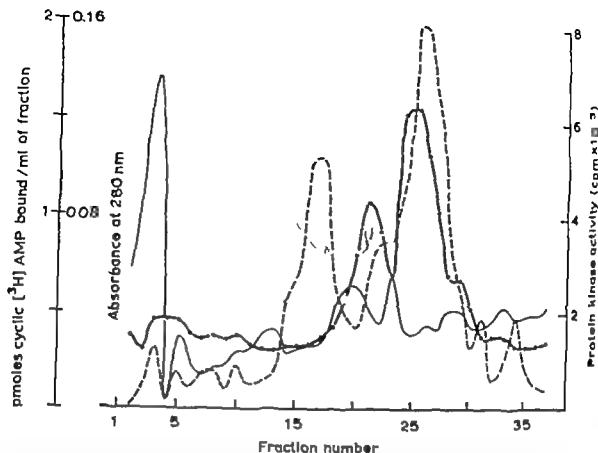


Fig 2 DEAE-cellulose chromatography. A column (0.9 x 15 cm) of DEAE-cellulose was equilibrated with 10 mM Tris HCl pH 7.5 containing 4 mM EDTA and 6 mM 2-mercaptoethanol. Elution was with a linear gradient from 0.03 M KCl in the equilibration buffer. The gradient was started at Fraction 3. The symbols are as in the legend to Fig 1 except for absorbance at 280 nm, which is represented by a thin, solid line.

differentiated squamous carcinomas, one as a poorly-differentiated carcinoma, and two were composed of squamous and adeno-carcinomatous elements.

The pieces of tumour used for biochemical and karyological investigations were taken from the main part of the tumour and it was ensured that biopsies from the periphery of the pieces contained only tumour tissue. For study of antigen sections were taken both from the main part and the periphery (Fig. 3) of tumours.

Tests for Immunological Specificity of the Methods Used to Detect Antigen in Tissue Sections from Cervix Carcinoma

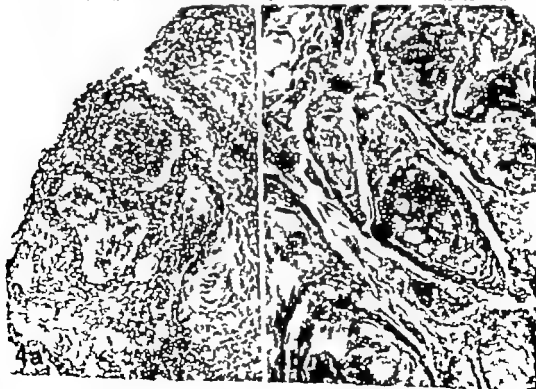
The sections appeared to be virtually free from indicator corpuscles or fluorescence when they had been incubated with pre-immune serum instead of immune serum. Only red corpuscles coated with both amboceptor and goat anti-rabbit antibody adhered to the sections. Furthermore, the fluorescence could be abolished by prior incubation of the immune serum with a partially purified preparation of CVA.

Occurrence and Localization of CVA in Carcinomas of the Cervix

The five first tumours were studied only by immunofluorescence. The seven following tumours were tested for CVA content by both immunofluorescence and mixed hemagglutination. Each of the two series contained one tumour in which immunofluorescence was barely detectable. The other ten tumours had areas of obvious immunofluorescence. Cervicovaginal antigen was detected in all the tumours studied by mixed hemagglutination. Eleven out of 12 tumours thus contained CVA, and it is possible that the tumour which was without

Fig 3 Histologic appearance of a cervix carcinoma. Section from the periphery of a tumour showing an early phase of tumour growth. Magnification, 50 x.

Fig 4 Association of indicator red blood corpuscles with the centres of nests of squamous epithelium directly below the surface (A) or in the periphery of the tumour (B). The anti CVA serum was diluted 1/50 000. Section fixed in formaline and stained with H.E. Magnification. A 120 X B 160 X.



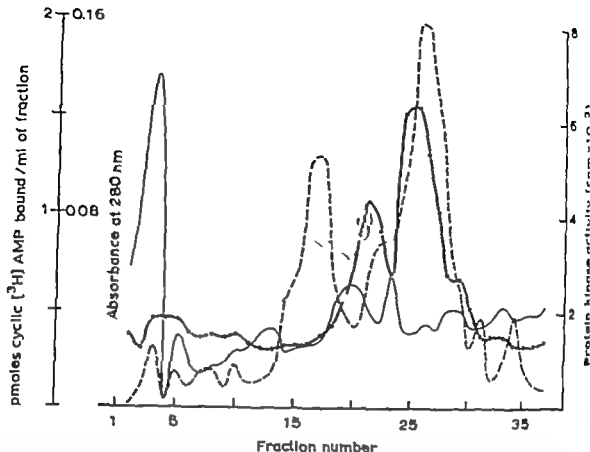


Fig 2 DEAE-cellulose chromatography. A column (0.9 x 15 cm) of DEAE-cellulose was equilibrated with 10% Tris-HCl pH 7.5 containing 4 mM EDTA and 6 mM 2-mercaptoethanol. Elution was with a linear gradient from 0.3 M KCl in the equilibration buffer. The gradient was started at Fraction 3. The symbols are as in the legend to Fig 1 except for absorbance at 280 nm, which is represented by a thin, solid line.

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lation phase of the cervicovaginal epithelium (13).

The highest concentrations of CVA were found in the morphologically more differentiated areas of the tumour, but high concentrations of the antigen could also be found in association with apparently isolated carcinoma cells infiltrating the connective tissue in the periphery of the tumour. The latter observation shows that an ordered alignment of the cells is not required for the production of CVA.

It also suggests that the generally rapid proliferation of the peripherally infiltrating carcinoma cells (1) and the production of a differentiated cell product like CVA are not mutually exclusive.

This work was supported by grants from the Norwegian Research Council for Science and the Humanities (NAVF) and the Norwegian Cancer Society (Lundformningen mot Kreft).

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FLUORESCENT LABELLING OF CELL MEMBRANES AND CYTOPLASMIC PROTEINS IN LIVING CELLS

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Fox, C. H., Auer, G., Zetterberg, A., Willems, J. S. & Locklund, B. Fluorescent labelling of cell
membranes and cytoplasmic proteins in living cells. *Acta path. microbial scand. Sect. A*, 86 131-
133 1978

Primary amino groups may be selectively labelled in living cell cytoplasmic components by staining
with the covalently binding fluorochrome reagent fluorescamine. The reaction is extremely rapid and
occurs at very low reagent concentrations. Cells survive such treatment and gradually remove or
metabolize the labelled substances. Nuclei and nucleoli are not labelled, while lamellar cytoplasm,
which contains little actual cytoplasm, is well demonstrated, thus indicating that the method is useful for
studies of cell membrane components. Labelled cell membranes can be prepared for further purification
after preliminary external fixation of cell membranes with a supravital polyaldehyde fixative. The use
of dimethylsulfoxide as a solvent for fluorescamine allows much longer survival of the cells than the
use of acetone as a solvent. In addition, the use of high pH and borate buffer was not necessary to the
labelling phenomenon.

Key words: Cell membranes, cytoplasmic proteins, fluorescent labelling.

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Received 28 vi 77 Accepted 14 x 77

The introduction of fluorescamine (Fluorim®)
Roché Diagnostic) as a method for fluorescent detection
of primary amines has allowed their measurement in
very small amounts in proteins, peptides, amino acids
and polyamines. One of the more interesting properties
of the compound is that it reacts with primary amino
groups very rapidly to form fluorescent compounds, and
with water to form spontaneous degradation products.
L. Jeunin *et al.* (1972) have suggested that fluorescamine
has a reaction half time with primary amines of 100-500
msec and with water of 5-10 seconds. These kinetics
suggested to us that it might be possible to use
fluorescamine for an evaluation of cell proteins at or near
the cell surface. Recently Henkens and Bartholomew
(1977) have reported results of labelling cells with
somewhat different method than that reported here, but
failed to comment on the survival of cells after the
labelling period. Since labelled cell membranes have
considerable potential for use in cell fusion experiments
we felt it to be interesting to investigate the biological
effects of staining cells with fluorescamine.

MATERIALS AND METHODS

Cells. Primary cultures of mouse renal epithelial cells
were obtained by removing kidneys from 10-14 days
old white mice, trypsinization for 16-24 hours in the
cold (4 °C), and subsequent trypsinization at 37° C for
an hour before gentle filtration and plating onto Barker
haemocytometer cover glasses in plastic petri dishes. The
cell clumps so obtained were allowed to attach and
flatten on the glass substrate for various periods in
Eagle's minimum essential medium containing 10% heat
inactivated calf serum (Gibco). The petri dishes were
incubated in an atmosphere of 10% CO₂ at 37° C.

Cultures of 3T3 cells were obtained from Chemoform
AB (Gibco-Bio-Cult) and grown on Barker cover glasses
under the same conditions. Stock cultures were passaged
at subconfluence, but no effort was made to follow the
3T3 regimen (Fadure & Green 1963).

Solutions of fluorescamine were prepared by weigh-
ing out the dry powder and dissolving it in dry acetone
or DMSO (Merck). Solutions were non-fluorescent in
visible light. Cells were prepared for reactions by

washing in two changes of Earle's basal salts solution. In a vessel containing 10 ml of salt solution and eight Burkner glasses (26 x 21 mm each), 0.5 ml of fluorescamine solution was added by forceful ejection from a tuberculin syringe. The solutions were rapidly mixed and allowed to incubate for thirty seconds after which the cover glasses were examined directly by phase- and/or fluorescence microscopy fixed in 6.5% cacodylate buffered glutaraldehyde (pH 7.3-0.2 M) or removed to Earle's MEM + 10% calf serum for further incubation.

For preparation of labelled cell membranes, cells were grown to confluence in a plastic T 25 flask and removed by use of a rubber policeman. The harvested cells were then washed in two changes of Earle's buffered saline (pH 7.4) and the outer membranes were fixed with the multi-aldehyde fixative developed by Phillips *et al* for 30 minutes. The cells were washed again with two changes of Earle's saline and reacted with fluorescamine in DMSO (0.5 ml) while suspended in 2.0 ml of saline. They were washed again and the pellet was suspended in 10.0 ml of 0.004 M Tris buffer. This treatment caused the cells to break open and the resulting membranes could be separated and further concentrated by sucrose density gradient centrifugation as described by Warren (p. 159).

RESULTS AND DISCUSSION

Cells were clearly marked using the procedure outlined above. When viewed with the fluorescence microscope, the cytoplasm of the cells was intensely fluorescent, even areas of lamellar cytoplasm where there are only low concentrations of mass or protein. Occasionally cells could be found that showed a concentration of fluorescence over the nucleus, sometimes directly over the nucleolus, but this was not always the case (Fig. 1). The pattern of fluorescence over the cell was usually granular with a much lighter background of faintly fluorescent cytoplasm.

Concentrations of fluorescamine in the reaction mixture was not a critical factor. Roughly the same amounts of fluorescence were obtained if the concentration of fluorescamine was varied between 7.5 μ g and 150 μ g per ml of final reaction mixture. The amounts of fluorescamine incorporated into proteins may depend in part on the very limited solubility of the compound in water.

In interphase cells, only the cytoplasm was stained, which might have been due to different permeability of the nuclear membrane, but in dividing cells that had just completed metaphase the chromosome mass was not stained, a finding we interpret to indicate that the reagent penetrates only the periphery of the cell. Håkanson *et al* has found that even in fixed cells there is little nuclear staining which may indicate there is a barrier to fluorescamine at the nuclear membrane, even in fixed cells. Hawkes *et al* (1976) did not find that cytoplas-

mic enzymes were labelled by fluorescamine or at least at a level detectable by their methods. On the basis of photographic and visual evidence we believe that there is some penetration of the cell membrane and cytoplasmic staining and for this reason, it is important to employ fixation of membranes before their isolation.

Membranes produced by this method were clearly marked after rupture of the intact cells but difficult to determine the purity of the membranes from cytoplasmic debris.

The best solvent for application of fluorescamine was found to be dimethylsulfoxide. The compound is very soluble and is well tolerated by the cells. When acetone was substituted in its place with



Fig. 1 Living culture of mouse kidney cells that have been stained with fluorescamine. Fig. 1a shows cells photographed with phase optics and Fig. 1b shows fluorescence of cells. Original magnification 250X.

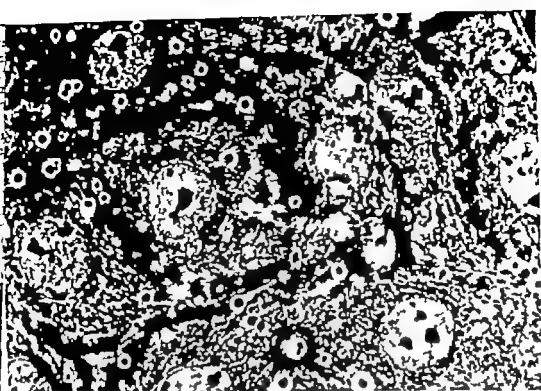


Fig 2 Mouse kidney cells 24 hours after application of fluorescamine in DMSO. The cells have normal morphology and show no evidence that the treatment has resulted in significant cell death. 900X

final concentration of 7.5 μ g of fluorescamine and an acetone concentration of 80.6 μ g per ml, cells incubated a further six hours after reaction showed indications of toxicity including increased cytoplasmic granularity, membrane retraction and rounding up of the cells. Prominent fluorescent granules were evident in the cytoplasm. Because of the toxic effects of acetone, we routinely used a final concentration of 122 μ g of DMSO with 7.5 μ g/ml of fluorescamine to stain living cells.

The principal disadvantage of this staining method is the rapid fading of fluorescence. We are investigating flow and short exclusion times for quantitative applications to membrane measurement.

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The principle disadvantage of this staining method is the rapid fading of fluorescence. We are investigating slow and short excitation times for quantitative applications to membrane measurement.

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THE EFFECT OF TOBACCO SMOKE CONDENSATE ON THE GROWTH AND LONGEVITY OF HUMAN DIPLOID FIBROBLASTS

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Litwin J, Enzell C & Pilotti Å. The effect of tobacco smoke condensate on the growth and longevity of human diploid fibroblasts. Acta path. microbiol. scand. Sect. A, 86: 135-141, 1978.

Human embryonic diploid lung fibroblasts were exposed to various fractions of cigarette smoke condensate over their *in vitro* life-time. Most fractions were toxic at a concentration of 50 µg/ml, with the exception of the strong acid and one water-soluble basic fraction, which stimulated growth and increased longevity significantly at this concentration. Most fractions produced no effect on cell growth at 10 µg/ml, with the exception of another basic fraction which inhibited growth at 1 µg/ml. Nicotine had no apparent effect on growth and longevity at 50 µg/ml. The neutral fraction containing the polynuclear hydrocarbon carcinogens produced normal growth and longevity at 10 µg/ml. No cell transformations were observed.

Key words: Tobacco smoke condensate, human diploid fibroblasts.

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Received 23 x 77 Accepted 15 x 77

At least 2350 chemical substances have been detected in cigarette smoke (27); they include carcinogens, anti-carcinogens (6, 16, 35) and various substances which inhibit enzyme activity (1, 7, 10, 25, 41) and influence DNA synthesis and repair (5, 11, 18, 19, 20). Cellular reactions *in vitro* to this complex mélange of chemical substances vary considerably depending on the type of cell studied. Macrophages and lymphocytes appear to be most sensitive to the toxic or inhibitory substances in cigarette smoke (15, 25, 76, 41), while fibroblasts, epithelial and endothelial cells are relatively resistant (38), although the cells growing from explants of human and hamster lung-tissue have shown considerable variation in DNA content and karyology (17, 18, 19, 20, 34). *In vitro* these effects usually appear within a few days following exposure to cigarette smoke. However, adverse

effects *in vivo* due to smoking usually do not become apparent before many years of intensive cigarette smoking. Thus, to simulate the *in vivo* situation best, an *in vitro* cell culture system was used which permitted continuous long-term exposures to cigarette smoke condensate (CSC). Since human diploid fibroblasts have a limited life-expectancy *in vitro* (12, 13) and undergo morphological and biochemical changes with time, a process associated with cell ageing (12, 13, 24), they were selected for such long-term experiments.

Previous studies by Swan (34) had shown that the serial propagation of human lung fibroblasts was only slightly influenced by 25 µg/ml of a water-soluble extract of cigarette smoke, while growth was inhibited by 50 µg/ml. No cell transformations were observed. The results presented in the present publication agree with those of Swan (34) and extend these observations by

RESULTS

ethanol for subsequent dilutions were made in methanol, which was less inhibitory to cell growth than ethanol. The final concentration of alcohol in the culture medium did not exceed 0.5%. The fractions were stored in dark bottles at -20°C . The dilutions were made when needed and then discarded. Nicotine was purchased from Sigma Chem. Co.

The first experiment was performed with whole CSC and the derived TA, TB and N fractions (Figs. 1-4). The two controls in Fig. 1 had longevity of 39.9 and 34.7 population divisions. Generally controls varied in longevity by ± 5 population divisions from the mean.



Fig. 1 Eagle's MEM containing 0.5% methanol C—C (61%), C—C (51%) Whole cigarette-smoke condensate 10 $\mu\text{g}/\text{ml}$ O—O (53%), 1 $\mu\text{g}/\text{ml}$ ●—● (71%), 0.1 $\mu\text{g}/\text{ml}$ △—△ (37%).

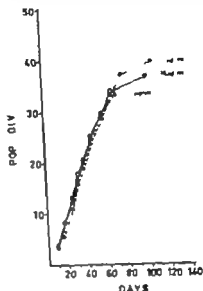


Fig. 3 Basic fraction 10 $\mu\text{g}/\text{ml}$ O—O (68%), 1 $\mu\text{g}/\text{ml}$ ●—● (66%), 0.1 $\mu\text{g}/\text{ml}$ △—△ (52%).

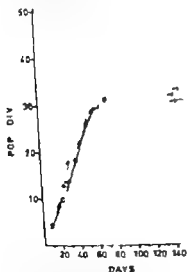


Fig. 2 Neutral fraction 10 $\mu\text{g}/\text{ml}$ O—O (54%) (concentrated as recommended) 1 $\mu\text{g}/\text{ml}$ ●—● (58%), 0.1 $\mu\text{g}/\text{ml}$ △—△ (48%).

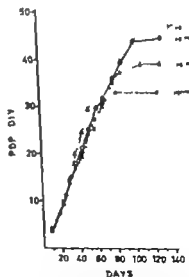


Fig. 4 Acid fraction 50 $\mu\text{g}/\text{ml}$ X—X (47%), 10 $\mu\text{g}/\text{ml}$ O—O (62%), 1 $\mu\text{g}/\text{ml}$ ●—● (56%), 0.1 $\mu\text{g}/\text{ml}$ △—△ (49%).

Figures 1-4 The growth of HEDLF cells in the presence of cigarette smoke condensate and the neutral, basic and acid fractions. The ordinate is in population divisions and the abscissa in days. Each point on the curves represent one passage. All fractions were toxic at 100 $\mu\text{g}/\text{ml}$. The values in parenthesis in the figures legends represent the average cell attachment for all of the serial transfers of these cells *in vitro*.

studying the effect of various fractions of CSC on the growth and longevity of human embryonic diploid lung fibroblasts (HEDLF)

METHODS AND PROCEDURES

Cell strain The HEDLF cell strain was grown out from human embryonic lung tissue in this laboratory. The cells were frozen after approximately 15 population divisions *in vitro* and stored in liquid nitrogen. All experiments were started from this supply. The karyology and growth characteristics were normal and similar to cells described by Hayflick & Moorhead (12) and Hayflick (13).

Medium Eagle's minimum essential medium (MEM) was used for all cell cultures, supplemented with 4 mM glutamine, 1 mM Na pyruvate, 10% calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 U/ml mycostatin, 1 µg/ml fungazone and 10 µg/ml aureomycin (23). The serum was not heat inactivated.

Passage procedure The cells from one frozen ampule were seeded into a Roux bottle containing 250 ml MEM. When the cell layer became confluent the cells were suspended in 0.25% trypsin in Hanks buffer, centrifuged and distributed into small culture bottles (growth surface 46 cm²) containing 20 ml MEM. The cells were allowed to grow out to a confluent monolayer. Bottles showing equally good growth were selected, and the cells were trypsinized, pooled and counted. Two hundred thousand cells were added back to each bottle together with 20 ml MEM and the substance to be tested. The same bottle was re-used throughout the experiment without intermittent washing. This procedure avoided variations in growth due to dish washing and had no influence on diploid cell growth or longevity.

When the cultures became confluent the cells were trypsinized, counted with an electronic cell-counter (Celloscope, Sweden) and 2×10^5 cells were added back to the same bottle. The following day the number of cells which attached to the glass was estimated with the aid of an inverted microscope (22, 23). The number of population divisions which occurred during a passage was calculated using the number of cells attached to the glass as the starting concentration. The percentage of cells attached for every passage in each series was averaged, and this value is given in the figure legends and Table 2. A comparison of the average percent cell attachment from the control cultures of many independent experiments has shown that the variation lies within $\pm 20\%$ of the average value. The range of cell attachment over the passage series usually ranges from 100% to 35%. Generally the attachment decreases markedly as the cell population approaches senescence.

The medium was changed once a week in case the cultures were not passed. The pH was maintained at about 7.5 by the periodic addition of Na bicarbonate.

When the cells became senescent and stopped growing the cultures were maintained for about one month before being discarded.

Smoking and fractionation procedure Ten thousand commercially available 85 mm nonfilter blend cigarettes

TABLE 1 The Percentage by Weight of the Various Fractions in Cigarette Smoke Condensate

Fraction	% of total condensate
N	23.10
B _{1a}	0.60
B _{1b}	0.91
B ₂	2.71
B	4.77
WA ₁	3.14
WA ₂	3.07
SA ₁	1.99
SA ₂	3.67
SA ₃	48.85

The fractions contain the following types of substances: N - hydrocarbons, polynuclear aromatic hydrocarbons, aldehydes, ketones, alcohols, terpenes, esters; B_{1a}, B_{1b}, B₂, B₃ - aliphatic and aromatic amines, pyridines, pyrazines, nicotine (> 90%);

WA₁, WA₂ - phenols;

SA₁, SA₂, SA₃ - strong phenols.

were conditioned at 65% relative humidity, 25°C, and smoked on an automatic Borgwald machine, using standard smoking parameters (pull volume 35 ml, pull duration 2 sec, pull frequency 1 puff/min, butt length 23 mm).

The smoke was trapped in a cooled condenser (CD, ethanol) and the cigarette smoke condensate (CSC) was removed from the trap with acetone. After removal of the solvent *in vacuo* at low temperature, the CSC was immediately subjected to separation into subfractions. The fractionation and further handling of the subfractions were in accordance with the procedure of Swann et al. (33). The percentage by weight of the various fractions in the total condensate is given in Table 1. The initial experiment was performed with total condensate (CSC) and three subfractions comprising total bases (TB), neutrals (N) and total acids (TA) which were obtained by a simplified fractionation procedure. After the initial partitioning of the CSC between ether and base, TA was obtained from the aqueous layer by extraction with ether at pH 1. Any precipitate (WA₁, SA₁) was also added to this ether phase. The remaining aqueous layer (SA₂) was concentrated; the residue was extracted with ethanol, and the ethanol extract was combined with the TA ether solution. Removal of the solvent yielded TA which consequently consisted of all weak and strong acids (WA₁, WA₂, SA₁, SA₂, SA₃). The neutrals and the bases in the first ether extract were separated in accordance with the original procedure (acidification, extraction with ether). Fraction N is identical in both fractionation sequences. After removal of N the remaining aqueous phase was extracted with ether at pH 11. This extract was combined with the precipitates and water-soluble bases, and evaporation yielded TB which was composed of B_{1a}, B_{1b}, B₂ and B₃.

All fractions were dissolved in small amounts of

ethanol but subsequent dilutions were made in methanol which was less inhibitory to cell growth than ethanol. The final concentration of alcohol in the culture medium did not exceed 0.5%. The fractions were stored in dark bottles at -20°C . The dilutions were made when needed and then discarded. Nicotine was purchased from Sigma Chemical Co.

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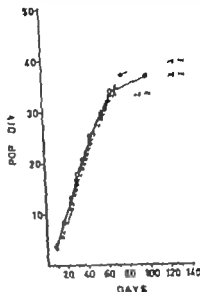


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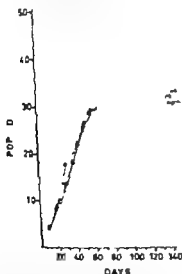


Fig. 2. Neutral fraction 10 $\mu\text{g}/\text{ml}$. O—O (54%) (contaminated at serialence), 1 $\mu\text{g}/\text{ml}$. ●—● (58%), 0.1 $\mu\text{g}/\text{ml}$. Δ — Δ (48%)

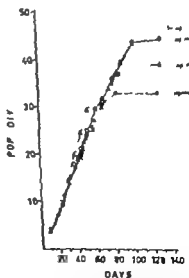


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Cell strain The HEDLF cell strain was grown out from human embryonic lung tissue in this laboratory. The cells were frozen after approximately 15 population divisions *in vitro* and stored in liquid nitrogen. All experiments were started from this supply. The karyology and growth characteristics were normal and similar to cells described by Hayflick & Moorhead (12) and Hayflick (13).

Medium Eagle's minimum essential medium (MEM) was used for all cell cultures, supplemented with 4 mM glutamine, 1 mM Na pyruvate, 10% calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 U/ml mycostatin, 1 µg/ml fungazone and 10 µg/ml aureomycin (23). The serum was not heat inactivated.

Passage procedure The cells from one frozen ampule were seeded into a Roux bottle containing 250 ml MEM. When the cell layer became confluent the cells were suspended in 0.25% trypsin in Hanks buffer, centrifuged and distributed into small culture bottles (growth surface 46 cm²) containing 20 ml MEM. The cells were allowed to grow out to a confluent monolayer. Bottles showing equally good growth were selected and the cells were trypsinized, pooled and counted. Two hundred thousand cells were added back to each bottle together with 20 ml MEM and the substance to be tested. The same bottle was re-used throughout the experiment without intermittent washing. This procedure avoided variations in growth due to dish washing and had no influence on diploid cell growth or longevity.

When the cultures became confluent the cells were trypsinized, counted with an electronic cell-counter (Celloscope, Sweden) and 2×10^5 cells were added back to the same bottle. The following day the number of cells which attached to the glass was estimated with the aid of an inverted microscope (2, 21). The number of population divisions which occurred during a passage was calculated using the number of cells attached to the glass as the starting concentration. The percentage of cells attached for every passage in each series was averaged and this value is given in the figure legends and Table 2. A comparison of the average percent cell attachment from the control cultures of many independent experiments has shown that the variation lies within $\pm 20\%$ of the average value. The range of cell attachment over the passage series usually ranges from 100% to 35%. Generally the attachment decreases markedly as the cell population approaches senescence.

The medium was changed once a week in case the cultures were not passed. The pH was maintained at about 7.5 by the periodic addition of Na bicarbonate.

When the cells became senescent and stopped growing, the cultures were maintained for about one month before being discarded.

Smoking and fractionation procedure Ten thousand commercially available 85 mm nonfilter blend cigarettes

TABLE 1 The Percentage by Weight of the Various Fractions in Cigarette Smoke Condensate

Fraction	% of total condensate
N	23.10
B _{1a}	0.60
B _{1b}	0.91
B ₂	2.71
B ₃	4.77
WA ₁	3.14
WA ₂	3.02
SA ₁	1.99
SA ₂	3.62
SA ₃	48.85

The fractions contain the following types of substances: N—hydrocarbons, polynuclear aromatic hydrocarbons, aldehydes, ketones, alcohols, terpenes, esters; B_{1a}, B_{1b}, B₂, B₃—aliphatic and aromatic amines, pyridines, pyrazines, nicotine (> 90%);

WA₁, WA₂—phenols;

SA₁, SA₂, SA₃—strong phenols.

were conditioned at 65% relative humidity, 35°C, and smoked on an automatic Borgwald machine, using standard smoking parameters (pull volume 35 ml, pull duration 2 sec, puff frequency 1 puff/min, but length 23 mm).

The smoke was trapped in a cooled condenser (CO₂/ethanol) and the cigarette smoke condensate (CSC) was removed from the trap with acetone. After removal of the solvent *in vacuo* at low temperature, the CSC was immediately subjected to separation into subfractions. The fractionation and further handling of the subfractions were in accordance with the procedure of Swann & al (33). The percentage by weight of the various fractions in the total condensate is given in Table 1. The initial experiment was performed with total condensate (CSC) and three subfractions comprising total bases (TB), neutrals (N) and total acids (TA), which were obtained by a simplified fractionation procedure. After the initial partitioning of the CSC between ether and base, TA was obtained from the aqueous layer by extraction with ether at pH 1. Any precipitate (WA₁, SA₁) was also added to this ether phase. The remaining aqueous layer (SA₂) was concentrated; the residue was extracted with ethanol, and the ethanol extract was combined with the TA ether solution. Removal of the solvent yielded TA, which consequently consisted of all weak and strong acids (WA₁, WA₂, SA₁, SA₂, SA₃). The neutrals and the bases in the first ether extract were separated in accordance with the original procedure (acidification, extraction with ether). Fraction N is identical in both fractionation sequences. After removal of N the remaining aqueous phase was extracted with ether at pH 11. This extract was combined with the precipitates and water-soluble bases, and evaporation yielded TB, which was composed of B_{1a}, B_{1b}, B₂ and B₃.

All fractions were dissolved in small amounts of

fractions were toxic at 50 $\mu\text{g}/\text{ml}$ with the exception of B₄, SA₁, SA₂ and SA₄, while 10 $\mu\text{g}/\text{ml}$ usually permitted normal growth and longevity. Fraction B₁₀ was toxic at 10 $\mu\text{g}/\text{ml}$ and inhibitory at 1 $\mu\text{g}/\text{ml}$, while TA₁ was inhibitory at 10 $\mu\text{g}/\text{ml}$.

Fractions B₄, SA₂ and SA₄ showed stimulation of growth and longevity at 50 $\mu\text{g}/\text{ml}$ which decreased at lower concentrations. Fraction SA₁ yielded normal growth at 50 $\mu\text{g}/\text{ml}$, but a stimulation at lower concentrations.

Most of the fractions appeared to have no influence on the average attachment of cells onto the glass with each passage except when applied at inhibitory concentrations. The one exception was TA₁ at 50 $\mu\text{g}/\text{ml}$, with an average attachment of only 34%. The attachment increased upon dilution, which implies the presence of an inhibitor.

No cell transformation was observed in any of the passage series.

DISCUSSION

CSC is a highly complex mixture of chemicals which produce a wide array of responses *in vivo* and *in vitro*. Studies on mice have suggested that CSC is a very weak initiator of tumors but has considerable promoting activity in mice pre-treated with initiators such as 7,12-dimethylbenzo[*a*]anthracene or benzo[*a*]pyrene (2, 3, 4, 14, 30, 31, 35, 36, 39, 40). The neutral fraction of CSC containing the polynuclear aromatic hydrocarbon carcinogens, has all of the initiating activity and much of the promoting activity (2, 3, 14, 31). The acid or phenolic fractions also display important promoting activity (2, 3, 14, 30, 39). The presence of reinitiating substances has also been reported (6, 16, 35).

Along with the general toxic or growth inhibitory activity of cigarette smoke on various types of cells *in vitro* (11, 15, 17, 18, 19, 20, 26, 41), the transforming activity of CSC has been demonstrated in mouse, rat and hamster embryo cell lines which had been infected with C-type RNA viruses (8, 9, 18, 29). The tumor-producing activity in mice required a large number of applications of CSC over long periods of time, whereas cell transformation in the virus-infected cell-culture system required a relatively short time for expression.

Human diploid fibroblasts were selected as the test system because previous investigations had shown that cell-aging is a very sensitive indicator of subtle changes in the cellular environment which may not be reflected in rate of growth (22, 23). Sato (24) showed that concentrations of water-soluble CSC greater than 25 $\mu\text{g}/\text{ml}$ were toxic to human fibroblasts, and lower concentrations had little effect on these cells. However, Srikant & van Dierck (32) demonstrated that although 1 $\mu\text{g}/\text{ml}$ CSC was non-inhibitory in mass culture of 3T3

mouse fibroblasts, their cloning efficiency was reduced significantly. This report suggests that population density may be an important factor in interpreting cell toxicity. No attempt to study the cloning efficiency of treated cells was made, because the value is low for diploid fibroblasts and it is difficult to estimate in ageing cell populations (21). In our cultures the original cell density at the start of each passage varied from between 4.3×10^3 cells/cm² (100% attachment) and about 1.4×10^3 cells/cm² (35% attachment).

The present results showed that at 10 $\mu\text{g}/\text{ml}$ CSC and most of its subfractions had no obvious effect on cell growth even though the fractions used were much more concentrated relative to CSC (N = 4 fold concentrated, B₁ = 200 fold, B₁₀ = 100 fold, B₄ = 40 fold, B₆ = 20 fold, TA₁ = 35 fold, TA₂ = 35 fold, SA₁ = 50 fold, SA₂ = 33 fold, SA₄ = 2 fold). Therefore, concentrations of fractions B₄, SA₂ and SA₄ which produced the greatest increase in cell longevity at 50 $\mu\text{g}/\text{ml}$, correspond to highly toxic concentrations of the total condensate. Possible synergistic effects by combining fractions were not studied.

The polynuclear aromatic hydrocarbon carcinogens (N fraction) had no effect on cell longevity nor did they promote cell transformation. These results may be due to the lack of a metabolizing system and oncogenic viruses in these cells.

The slight reduction in longevity produced by the TB fraction (Fig. 3) and the toxic effect of one of its subfractions (B₁₀, Table 2) cannot be explained by the presence of nicotine, which constitutes about 90% of TB. Nicotine showed little or no effect on these cells (Table 2).

The growth-stimulatory effect of TA (Fig. 4) appears to be due mainly to the strong acid fraction. Since the acid fraction of CSC has been reported to contain tumor-promoting activity in mice (3, 4, 14, 30, 39) it is possible that the increased longevity in human diploid fibroblasts was produced by such chemicals. The fractions used in these studies were too crude to allow any definitive interpretation.

Unfortunately cell-aging *in vitro* is a factor about which little is known, and its significance in terms of *in vivo* observations is obscure. Nevertheless, the fact that the serious deleterious effects of smoking appear in people who have smoked heavily for many years suggests that long exposures to high concentrations are necessary to produce these effects. Since the substances which increased cell longevity were also active at high concentrations, there may be some relationship between these observations.

Further attempts to identify the chemical entities which increase cell longevity may shed some light

At 100 $\mu\text{g/ml}$ the CSC TB and N fractions were toxic, whereas cells exposed to 100 $\mu\text{g/ml}$ TA underwent about 4 divisions and degenerated after one month's incubation.

At 10 $\mu\text{g/ml}$ and lower concentrations of CSC and the N fraction the cells growth and longevity were similar to the controls (Figs. 1 and 2) whereas

TB reduced cell longevity slightly but had no influence on the growth rate (Fig. 1).

The acid fraction increased longevity significantly at 50 and 10 $\mu\text{g/ml}$ (Fig. 4) without affecting the growth rate; the longevity decreased with dilution.

This experimental procedure was repeated, using more extensively fractionated CSC (Table 2). Also

TABLE 2 The Effect of Cigarette Smoke Condensate Fractions on the Growth and Longevity of HEDLF Cells

Sample	Total no cell div	Cell div at senesc.	Time senesc. occurred (days)	Cell div days	Total time (days)	Ave % cells attached
Eagles control	34.5	34.0	85	0.40	111	56
0.5% ethanol	37.5	29.6	68	0.44	93	52
0.5% methanol	34.2	34.7	76	0.45	111	70
50 $\mu\text{g/ml}$ nicotine	30.7	30.7	76	0.40	93	54
10 $\mu\text{g/ml}$ nicotine	37.2	33.9	68	0.50	93	60
1 $\mu\text{g/ml}$ nicotine	38.1	35.2	68	0.52	93	75
50 $\mu\text{g/ml}$ CSC	3.7	—	—	—	27	18
10 $\mu\text{g/ml}$ CSC	33.3	32.3	76	0.43	111	59
1 $\mu\text{g/ml}$ CSC	41.3	40.7	83	0.49	111	64
50 $\mu\text{g/ml}$ N	3.8	—	—	—	43	31
10 $\mu\text{g/ml}$ N	38.4	34.9	68	0.51	93	61
1 $\mu\text{g/ml}$ N	77.9	—	—	0.52	54	76
50 $\mu\text{g/ml}$ B ₁	4.3	—	—	—	43	36
10 $\mu\text{g/ml}$ B _{1a}	28.1	—	—	0.52	54	64
1 $\mu\text{g/ml}$ B _{1a}	38.0	35.0	67	0.57	93	71
50 $\mu\text{g/ml}$ B _{1b}	Toxic	—	—	—	—	—
10 $\mu\text{g/ml}$ B _{1b}	Toxic	—	—	—	—	—
1 $\mu\text{g/ml}$ B _{1b}	18.6	—	—	0.35	54	43
50 $\mu\text{g/ml}$ B _{2a}	45.7	45.7	83	0.55	111	60
10 $\mu\text{g/ml}$ B _{2a}	38.8	35.1	62	0.57	93	70
1 $\mu\text{g/ml}$ B _{2a}	36.4	32.9	67	0.53	93	71
50 $\mu\text{g/ml}$ B _{2c}	3.6	—	—	—	43	36
10 $\mu\text{g/ml}$ B _{2c}	39.2	38.3	76	0.50	111	64
1 $\mu\text{g/ml}$ B _{2c}	37.4	35.0	67	0.56	93	61
50 $\mu\text{g/ml}$ W A ₁	3.4	—	—	—	43	28
10 $\mu\text{g/ml}$ W A ₁	34.7	31.1	67	0.46	110	65
1 $\mu\text{g/ml}$ W A ₁	28.0	—	—	0.57	49	57
50 $\mu\text{g/ml}$ W A _{2c}	2.3	—	—	—	43	38
10 $\mu\text{g/ml}$ W A _{2c}	9.7	—	—	0.18	54	30
1 $\mu\text{g/ml}$ W A _{2c}	35.1	32.6	87	0.53	93	84
50 $\mu\text{g/ml}$ S A ₁	31.3	77.5	57	0.48	93	34
10 $\mu\text{g/ml}$ S A ₁	41.5	39.4	68	0.58	93	54
1 $\mu\text{g/ml}$ S A ₁	40.0	36.8	62	0.59	93	64
50 $\mu\text{g/ml}$ S A _{2c}	48.8	48.3	97	0.50	111	51
10 $\mu\text{g/ml}$ S A _{2c}	44.6	42.0	76	0.55	93	63
1 $\mu\text{g/ml}$ S A _{2c}	38.1	38.1	76	0.50	93	66
50 $\mu\text{g/ml}$ S A _{2a}	47.6	43.7	83	0.53	176	62
10 $\mu\text{g/ml}$ S A _{2a}	44.8	42.8	76	0.56	93	66
1 $\mu\text{g/ml}$ S A _{2a}	35.6	35.5	76	0.47	111	69

* Calculated by dividing cell divisions in senescence by time senescence occurred. In case of contamination the total no. cell divisions was divided by the total time.

Culture terminated by contamination

* Bottle broken accidentally

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on this question and the possible mechanism(s) controlling cell ageing

This work was partially supported by a grant from the Swedish Tobacco Co. The authors wish to gratefully acknowledge the excellent technical assistance of Mrs. Annette Litwin and Mrs. Ann Björk.

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STUDIES ON THE RAT LIVER FOLLOWING IRON OVERLOAD

1 Fine Structural Appearance

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Hultcrantz, R. & Arbogh, B. Studies on the rat liver following iron overload. I. Fine structural appearance. *Acta path. microbiol. scand. Sect. A*, 86: 143-153 1978.

Iron overload of the rat liver following parenteral administration of Jectofer® (an iron sorbitol citric acid complex) was studied in the electron microscope. Abundant ferritin-like granules were present in parenchymal and Kupffer cells, partly free in the cell sap and partly concentrated in 3 types of membrane-bound organelles, with characteristic appearances. In the parenchymal cells these organelles consisted of lysosome-like structures, apparent autophagic vacuoles, and vacuoles lacking features linking them to specific cytoplasmic elements. Organelle-bound ferritinlike granules in the Kupffer cells were demonstrated as lysosome-like structures, in phagocytic vacuoles, and in tubular and vacuolar elements referred to as type 1 and type 2 bodies. No ferritin-like granules were observed in other cell types than parenchymal and Kupffer cells.

Key words: Liver, rat, iron overload, electron microscopy.

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Received 21.11.77 Accepted 21.1.78

Iron storage can occur in many organs. The liver is considered to be one of the main sites where iron accumulates, both under normal and pathological conditions (4, 5). Although reports on the fine structure of the liver parenchymal cells in iron overload have appeared (1, 3, 11, 20, 24, 29), there is no complete analysis of the iron storing cells available at present.

In this study a detailed morphological description of the appearance of liver cells engaged in iron storage following iron overload is given. Since adequate preservation is of utmost importance when considering the intracellular localization of iron pigment, we have striven to diminish or avoid artifacts such as swelling or shrinkage, by carefully controlling the fixation procedure (12).

In addition to elucidating the fine morphology, this study is intended to serve as a basis for studies of the long term effects of iron overload and of the possibilities to mobilize the iron stores.

MATERIALS AND METHODS

Male Sprague Dawley rats (180-220 g) were given intramuscular injections of an iron sorbitol citric acid complex (Jectofer® Astra Pharmaceutical Company, Sodertälje, Sweden) 15 times during a three week period in a total dose of 50 mg Fe per 100 g of body weight (b.w.). The animals were kept in groups of four receiving standard laboratory diet and tap water *ad libitum* during the experimental period. No injections were given to the control animals.

The rats were sacrificed at 8 a.m. 24 hours after the last injection. The abdomen was cut open under light ether anaesthesia and heparin (0.1 mg/100 g b.w.) was administered in the left cardiac vein in order to prevent the formation of intravascular aggregates of erythrocytes in the liver during perfusion fixation. A catheter was inserted into the portal vein and secured with a ligature. Subsequently perfusion with saline (37°C) was started, after 10 seconds followed by the fixation agent (37°C, 3% glutaraldehyde, 0.1 M sucrose and 0.1 M cacodylate buffer) (22) which was continued for 10 min. In order to secure free flow of perfusate and fixative, the inferior

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portal vein had been cut open above and below the liver immediately after starting the perfusion.

Thin slices of liver tissue were then immersion fixed for 24 hours in the aforementioned glutaraldehyde medium (0-4° C) and postfixed for 2 hours in 2% *s* collidine buffered osmium tetroxide (0-4° C) for the electron microscope examination. Embedding was performed in Epon for electron microscopy and in paraffin for light microscopy. After cutting ultrathin sections and staining the sections with lead citrate, they were examined in a Jeol 100 C electron microscope.

The light microscope material was stained with Hematoxylin & Eosin (H&E). Perl's method was used for the demonstration of iron pigment.

RESULTS

Light Microscopy

No pathological changes were observed in the H&E stained liver tissue. After staining for iron pigment, the parenchymal cells showed presence of a finely granular reaction product, characteristically occurring more abundantly in the periportal areas of the lobule than centrilobularly. A heavy granular staining could also be observed in elongated nonparenchymal sinusoidal lining cells (probably Kupffer cells). Concerning this cell type, the "iron granules" were more frequent in the cells located near the portal areas than in cells near the center of the lobule (Fig. 1).

Electron Microscopy: Parenchymal Cells

The appearance of the parenchymal cells in control animals did not in any respect deviate from earlier descriptions. An example of the fine structure is given in Figure 3.

Iron overload induces accumulation of iron containing electron dense particles, hereinafter referred to as "LP" (iron particles). We have chosen to use this term since unanimous agreement as to the ultrastructural difference between hemosiderin and ferritin is lacking (9). In general these granules range from 30-120 Å units in largest diameter (6).

The particles were diffusely scattered throughout the cell sap occurring both inbetween cellular organelles (e.g. Golgi apparatus, endoplasmic reticulum and lysosomes) and intermingled with particulate glycogen (Figs. 4-9).

The highest concentration of LP was noted within single membranebound organelles usually located in the peribiliary regions where secondary lysosomes normally are seen (Fig. 2). Some of these iron loaded organelles clearly contained similar material as the secondary lysosomes in untreated

animals (Figs. 3-4). They are hence referred to as lysosomes in the figures. Other organelles contained tightly packed LP covering any possible matrix

Fig. 1 Light microscopy of liver tissue stained for ferrous iron. Iron granules are present in both Kupffer cells (arrows) and liver parenchymal cells. The staining is somewhat weaker near the central vein (V) than in other parts of the lobule. Glutaraldehyde. Paraffin sections stained with Perl's blue. $\times 300$.

Fig. 2 Low power electron micrograph showing part of liver tissue containing parenchymal (P) and Kupffer (K) cells. Iron-containing lysosomes are concentrated in the peribiliary regions of parenchymal cells. Lysosomes loaded with iron are also present in Kupffer cells. B, bile canaliculus; N, nucleus. Glutaraldehyde; OsO₄; Epon. Section stained with lead citrate. $\times 4,000$.

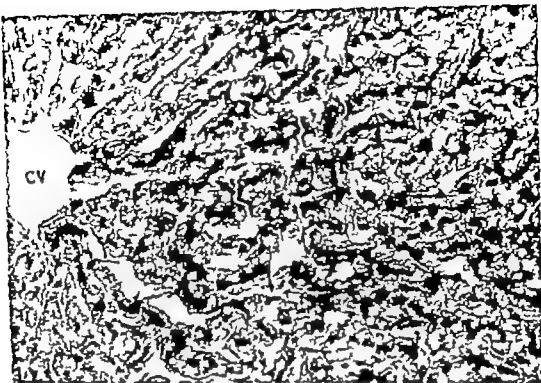
Fig. 3 Liver tissue from a control animal. Picture shows portion of a parenchymal cell near a bile canaliculus (BC). Ferritin is not readily identified in any of the cellular compartments. L, Lysosome; G, Golgi apparatus; ML, mitochondria; ER, endoplasmic reticulum; Gly, glycogen. Glutaraldehyde; OsO₄; Epon. Section stained with lead citrate. $\times 25,000$.

Fig. 4 Peribiliary regions of two parenchymal cells from an iron treated rat. All of the lysosomes (Ly) present are shown to contain iron particles LP in various amounts, some of them being heavily loaded. A few vacuoles (V) are seen containing nothing but LP. In the surrounding cytoplasm LP are evenly scattered. No LP can be seen in the Golgi apparatus (G), endoplasmic reticulum (ER) or in the mitochondria (ML). BC, bile canaliculus; Gly, glycogen. Glutaraldehyde; OsO₄; Epon. Section stained with lead citrate. $\times 25,000$.

Fig. 5 Picture shows two adjacent parenchymal cells surrounding a bile canaliculus (BC). Ferritin is present in numerous autophagic vacuoles (AV) containing glycogen and nonidentifiable matrix. Arrows indicate multivesicular bodies without LP. G, Golgi apparatus; Glutaraldehyde; OsO₄; Epon. Section stained with lead citrate. $\times 21,600$.

Inset shows a glycogen containing single membrane limited autophagic vacuole (AV) in the vicinity of a Golgi apparatus (G). Arrow indicates a probable fusion between a Golgi vesicle and the vacuole. No LP can be observed within the membrane limited organelles. Glutaraldehyde; OsO₄; Epon. Section stained with lead citrate. $\times 29,000$.

Fig. 6 Micrograph illustrating a parenchymal cell containing a great number of autophagic vacuoles (AV) with various amounts of LP. Observe that membranous material appear in some of the vacuoles (arrows). L, Lysosome; ER, endoplasmic reticulum. Glutaraldehyde; OsO₄; Epon. Section stained with lead citrate. $\times 23,100$.













by 7 Detail of a parenchymal cell where I.P. is accumulated within the lysosomes, some of which are ch in I.P. (inset). The Golgi apparatus (G) is well developed, containing no obvious ferritin particles. Glutaraldehyde, OsO₄ Epon. Section stained with lead citrate. x 33,200.

Inset showing high power micrograph of tightly packed I.P. in a lysosome. Glutaraldehyde, OsO₄ Epon. Section stained with lead citrate. x 70,000.

Fig 8 Picture illustrates the relation between rough endoplasmic reticulum and Golgi apparatus. Arrows indicate an area where continuity can be observed between the rough endoplasmic reticulum and a membrane bound lipoprotein containing vesicle. Double arrows indicate lysosome-like structures containing no visible I.P. Glutaraldehyde, OsO₄ Epon. Section stained with lead citrate x 25,000

Fig 9 A Golgi apparatus with clearly distinguished lipoprotein without I.P. Glutaraldehyde, OsO₄ Epon. Section stained with lead citrate x 26,500

Fig 10 Figure showing the spectrum of Danc (arrow) between a parenchymal cell and a Kupffer cell (Ka). No ferritin is seen extracellularly. Glutaraldehyde, OsO₄ Epon. Section stained with lead citrate x 29,000

Fig 11 Part of Kupffer cell showing different types of I.P. containing structures: (1) Large vacuolar structure with a rounded outline, (2) Slender tubular structures. A large phagocytic vacuole (V) containing globules of dense material and electron lucent matrix. Observe the thin border of I.P. on the inside of the membrane. I.P. can be frequently seen in the cytoplasm. Glutaraldehyde, OsO₄ Epon. Section stained with lead citrate x 33,000

Fig 12 Micrograph of Kupffer cell with a large, probably heterophagic vacuole (V) containing abundant glycogen and various cell organelles. An I.P. lining is noticed on the inside of the limiting membrane. Arrows indicate small I.P.-containing tubular structures in continuity with the vacuole. (1) rounded body containing densely packed I.P. (3), large organelle with regular outline and with an appearance of a Kupffer cell lysosome containing I.P. dense granular and membranous material. Double arrows show tubular structures containing I.P. Glutaraldehyde, OsO₄ Epon. Section stained with lead citrate x 18,500

Inset: Pattern of vacuole with I.P. concentrated at the periphery. Glutaraldehyde, OsO₄ Epon. Section stained with lead citrate x 29,000

Fig 13 Kupffer cell with three different types of I.P. containing structures: (1) densely packed I.P. in a rounded structure, (2) slender tubular structures, (3) lysosome-like structure. Glutaraldehyde, OsO₄ Epon. Section stained with lead citrate x 25,000

material; in the place of the section they lacked other identifiable materials than the I.P. Some of these bodies were located close to the Golgi apparatus (Fig. 4). In still other organelles, the matrix seemed to be divided into separate compartments composed of phagocytosed glycogen and I.P., respectively (Figs. 5 and 6). The latter organelles hence showed the appearance of autophagic vacuoles. Autophagic vacuoles with mitochondria, endoplasmic reticulum and other identifiable structures usually also contained I.P. No I.P. could be identified in the disternae and associated vesicles of the Golgi apparatus, the nucleus, the endoplasmic reticulum or the mitochondria. Likewise, no particles were noticed in the extracellular space between the parenchymal cells or in the bile canaliculi (Figs. 4-5-10).

Electron Microscopy Kupffer Cells

Kupffer cells in controls showed the same appearance as in previous descriptions. In iron loaded animals, large numbers of I.P. were present in this cell type. I.P. were scattered throughout the whole cell sap in a higher concentration than in the corresponding space of the parenchymal cell. There seemed to be no concentration differences in various portions of the cell sap. Three types of single membrane bound organelles contained densely packed I.P. These were: (a) Large vacuolar structures with rounded, irregular outline lacking identifiable features other than the I.P. These are referred to as type 1 bodies (Figs. 11-15), (b) slender tubular images, in cross sections appearing as vesicles and also exclusively filled with I.P. (Figs. 11-13), heretofore referred to as type 2 bodies, (c) usually large organelles with an irregular outline and containing dense, granular and membranous material with an appearance reminiscent of that in Kupffer cell lysosomes (Fig. 13). Small numbers of I.P. were found in occasional large phagocytic vacuoles (Fig. 12) and also in vacuoles with an electron lucent matrix containing occasional globules of dense material (Fig. 11). No I.P. were seen in the nucleus, the mitochondria or the endoplasmic reticulum. Some membrane-bound vesicular structures close to the Golgi apparatus (Fig. 15) did contain I.P. and might represent type 2 bodies, however no I.P. could be identified in the Golgi apparatus proper. I.P. were not observed outside the cell membrane (Figs. 10-17).

Electron Microscopy Endothelial Cells, and Fat Storing Cells

No I.P. could be identified in membrane bound structures or in the cell sap in the endothelial or the fat storing cells (Figs. 16-17).

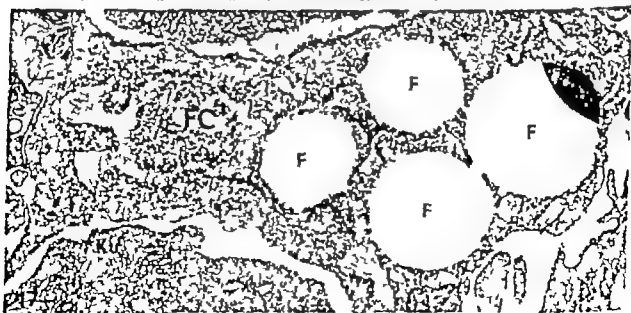


Fig 14 Picture showing slender bodies (2) containing I within a Kupffer cell. Arrows indicating a type (2) body surrounding part of the cell sap. (3) lysosomal-like containing body. Glutaraldehyde, OsO₄ Epon. Section stained with lead citrate. x 25,000

Fig 15 Part of Kupffer cell where a Golgi apparatus (G) can be observed. Single arrows indicate Golgi vesicles which do not contain any I P. Double arrows, a small vesicular structure adjacent to the Golgi apparatus and tightly packed with I P. Glutaraldehyde, OsO₄ Epon. Section stained with lead citrate. x 25,000

Fig 16 Micrograph showing an endothelial cell (EC). No I P can be seen in any part of the cell. Glutaraldehyde, OsO₄ Epon. Section stained with lead citrate x 18,000

Fig 17 Picture showing a fat storing cell (FC) with no fat containing vacuoles (F). No I P can be observed in the cell sap or in vacuolar tubular elements. For comparison, observe the abundant I P in the cytoplasm of a Kupffer cell (Kc) at the bottom of the picture. Glutaraldehyde, OsO₄ Epon. Section stained with lead citrate x 33,200.

DISCUSSION

In previous studies on iron overload in the rat, Pechet noted that iron is stored primarily in the liver parenchymal and Kupffer cells (24). Later storage also occurs in the cells of other organs such as the pancreas and the heart (20). Pechet also described enlargement and hyperplasia of the Kupffer cells in iron storage.

By light microscopy no pathological change other than the accumulation of iron positive granules was observed in the liver after the overload. This is in accordance with the experience of other investigators (1, 19, 20, 26).

The sections stained for ferrous iron revealed higher concentration of iron positive granules periporally than centrilobularly, a phenomenon previously described without any explanation. This distribution is noticed both regarding parenchymal and Kupffer cells and seems to be influenced by a mutual factor. Differences in fine structure between cells located close to, and far from, the central vein are also well documented (18, 23, 28). It is therefore possible that they like differences concerning the distribution of iron granules, are in some way related to the blood flow and oxygen tension in the tissue.

Arzuda et al. (3), in their work on iron storage in the rat liver parenchymal cells, present a condition they term "cellular iron overload". In excessively short time extracellular iron overload, the cells take up iron, incorporate it into apoferitin without

limiting steps, and store it in different compartments such as the lysosomal system, other vacuoles, and the cell sap. The greatest accumulation of ferritin in this model is in the lysosomes. The authors assume that degradative processes involving the transformation of ferritin into hemosiderin take place in these organelles. Since ferritin and hemosiderin do accumulate, it is suggested that the excretion of hemosiderin represents a limiting step in cellular iron metabolism, and is the cause of the cellular iron overload.

The synthesis of apoferitin can possibly be performed in different ways. One way would be on the free polysomes (14, 25); the synthesized protein might subsequently be autophagocytosed to become incorporated in the lysosomal system. Alternatively, synthesis might take place on the ribosomes of the rough surfaced endoplasmic reticulum. Apoferitin could then be transported through the channels of the ER and the Golgi apparatus to primary lysosomes (25).

In our study we have been using an iron sorbitol citric acid complex given intramuscularly. The molecular weight of this complex is approximately 5,000 and the size would then probably not permit other ways of uptake by cells than endocytosis. Subsequent to intramuscular injection, the bulk of the complex will reach the circulation, and in this way the parenchymal and Kupffer cells of the liver become exposed to the iron-containing molecules (17). Following their presumed uptake by the cells via endocytosis, the molecules are likely to become incorporated in the lysosomes like most other exogenous materials picked up by a variety of cells (13, 27). Degradation of the complex within the lysosomes may result in release of the iron moiety to the cell sap. A smaller portion of the iron deposited intramuscularly probably binds to transferrin and subsequently — via the general circulation — reaches the liver. This transferrin-bound iron can evidently be released at the plasma membrane of Kupffer and parenchymal cells, and in this way be directly incorporated into the cell sap.

Whichever way iron is transported to the cell sap, the result will be an increased synthesis of apoferitin (6, 8, 15, 21, 22). The binding of iron to apoferitin in the cell can occur in different locations such as the cell sap, the lumen of the endoplasmic reticulum, or elsewhere (10).

Parenchymal cells. The findings in the present study showed high concentrations of I P in several types of membrane-limited bodies. In the parenchymal cells, these bodies appeared to be lysosomes, autophagic vacuoles, and elements morphologically not clearly associated with these organelles, although the evidence suggests that they are lysosomes

cut in such a way that typical lysosomal matrix materials were not included or visible (7). If we assume that all the structures containing high concentrations of I.P. indeed are lysosomes, two ways for the assembly of the ferritin-like particles suggest themselves. In the case of autophagic vacuoles, ferritin molecules in the cell sap can be trapped during the formation of the vacuoles and in this way be directly enclosed in the forming lysosomal structure. Alternatively apoferritin travelling through the ER and/or the Golgi apparatus and binding iron to form ferritin could be channelled (in GERL) to lysosomes. A combination of these ways is not unlikely although we were not able to demonstrate presence of typical ferritin molecules in the cisternae of the ER or in the Golgi apparatus. Judging from the morphological appearances, it would seem that uptake by way of autophagy is one of the main means of assembly of the I.P. in lysosomes.

Kupffer cells This cell type has great capacity for phagocytosis and a well known ability to store iron bound to apoferritin (16). Also the same ways and regulation of synthesis and intracellular transport of apoferritin could be assumed for the parenchymal cells and the Kupffer cells.

I.P. were seen both in the cell sap and in membrane-bound structures in the Kupffer cells. The higher concentration of I.P. in the cell sap compared to the situation in parenchymal cells may be explained by the fact that Kupffer cells are more actively engaged in phagocytosis. They are also in closer and more direct contact with the sinusoidal blood than the parenchymal cell.

The main portion and the highest concentration of I.P. was seen in membrane-bound structures of different appearances. Some of these contained osmophilic and other electron dense material besides I.P. and they are likely to represent lysosomes. However other types of bodies also contained I.P. and their exact functional significance and nature remains obscure. These bodies are not found during ordinary conditions in the Kupffer cells of the rat. In order to clarify their relation - if any - to the lysosomal system, it is necessary to study their enzymatic content using histochemical methods.

We also found large vacuoles containing phagocytosed material having a thin border of I.P. on the inside of the bordering membrane. These organelles associated with extensive phagocytosing ability besides their function in storing I.P. are likely to correspond to enlarged lysosomes in which the I.P. have been diluted.

Endothelial and fat-storing cells did not show any I.P. indicating that they do not play any important

role in the process of iron storage during overload in the liver.

This study has been aided by grants from the Swedish Medical Research Council (Projects no 871-17 01006-12C and 13A).

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CONTRIBUTION TO THE KNOWLEDGE OF THE FINE STRUCTURE OF CHONDROSARCOMA OF BONE. WITH A NOTE ON THE LOCALIZATION OF ALKALINE PHOSPHATASE AND »ATPase«

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Aparisi, T. Arborgh, B. Ericsson, J. L. E. Göthlin, G. & Nilsson, U. Contribution to the knowledge of the fine structure of chondrosarcoma of bone. With a note on the localization of alkaline phosphatase and »ATPase«. *Acta path microbiol scand Sect. A*, 86: 157-167 1978.

Seven well differentiated chondrosarcomas of bone have been analyzed by electron microscopy and the fine structural localization of adenosine triphosphatase and nonspecific alkaline phosphatase has been elucidated. On the basis of the fine structural appearance, two distinct cell types were shown to constitute the tumor tissue: chondrocyte-like cells and large mitochondria-rich cells. Large, nonnucleated cells in the tumor did not seem to correspond to osteoclasts but rather were likely to represent true neoplastic cells. Some chondrocyte-like cells appeared to be binucleated by virtue of deep, groove-like nuclear indentations. Adenosine triphosphatase and alkaline phosphatase were associated with the plasma membrane of both chondrocyte-like and mitochondria-rich cells suggesting that they might be of common origin. Normal chondroblasts and chondrocytes lack histochemically demonstrable adenosine triphosphatase on their plasma membrane. Presence of this enzyme in the tumor cells may indicate that they are histogenetically related to immature non-chondroid matrix forming cells (known to carry the enzyme).

Key words: Bone tumor, chondrosarcoma, ultrastructure, alkaline phosphatase, ATPase

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Received 24 x 77 Accepted 24 x 77

Chondrosarcoma is the second most common primary malignant tumor of bone. At the light microscopic level, the tumor is rather well defined, whereas ultrastructural descriptions of the lesion are few and incomplete. The differentiation of chondrosarcoma is important because it is well correlated with the prognosis, this is in contrast to the situation with many other primary bone sarcomas where morphological differentiation appears to be of limited importance for the prognosis.

It seems likely that investigations concerning the cellular differentiation at the ultrastructural level will further extend our knowledge regarding the histogenesis and state of malignant potential, as well as prognosis in various cases of chondrosarcoma (3).

The application of enzyme cytochemical techniques to problems in oncology and cancer research has not until recently been extended to electron microscopy. The results in the comparatively few reports which have so far appeared concerning the

ultrastructural localization of enzymes in human neoplasms appear however to indicate that the prospect for further development in this field is encouraging.

The ultrastructural localization of phosphatases, such as adenosine triphosphatase (ATPase) and nonspecific alkaline phosphatase, in normal bone and cartilage tissue appears to be rather well documented.

By application of improved methods for preparation and incubation of normal hard tissues or bone tumor samples, it should be possible to obtain valuable information regarding modifications in the histochemical pattern of these enzymes in bone tumor cells as related to the situation in normal bone and cartilage cells. Even though this approach is still in a preliminary or data-collecting phase in morphologic cancer research one important concept has emerged namely that some neoplasms can be classified as to their cell or tissue of origin by detecting within the tumor a marker enzyme of the parent tissue.

In the present investigation the fine structure and localization (at the electron microscope level) of alkaline phosphatase and ATPase has been studied using previously established methodology (5, 6).

CASE HISTORIES

Case 1 (U.S.) Man, 57 years of age, with gradually increasing pain in the left shoulder region. X-ray examination showed a picture typical of chondrosarcoma of the scapula, this diagnosis being confirmed by needle biopsy. Removal of the tumor was carried out by scapulectomy. Histopathological examination of the resected specimen showed a well-differentiated chondrosarcoma. Two years later there are no signs of recurrence or metastases.

Case 2 (U.E.) Man, 54 years of age, with a painless palpable mass in the distal part of the right thigh. X-ray examination demonstrated an osteolytic process in the femur extending into the soft tissue. Open biopsy gave the diagnosis of a well-differentiated chondrosarcoma. Amputation was advised, but the patient refused and disappeared.

Case 3 (A.S.) Woman, 65 years of age, with a history of low-back pain and right-sided sciatica. X-ray examination revealed an osteolytic destruction of the body of the 4th lumbar vertebra. Needle biopsy gave the diagnosis of chondrosarcoma. The patient was operated upon in two stages. At the first stage a laminectomy was carried out on the 4th lumbar vertebra, followed by posterior fixation with two Harrington rods. At the same time, tissue was curetted from the vertebral body and the diagnosis of well-differentiated chondrosarcoma was histopathologically verified. At the second-stage operation the main part of the body of the 4th lumbar vertebra, including the tumor, was resected. The defect was filled with a block of bone-cement. More than one

year after the operation, the patient is symptom-free; there are no signs of local recurrence or metastases.

Case 4 (K.L.) Man, 19 years of age, with pain in the right groin upon sport activities. X-ray examination demonstrated a calcified mass originating from the right pubic bone and extending behind the symphysis and dislocating the urinary bladder. Open biopsy was performed twice. On one occasion the histopathology of the resected tissue was interpreted as well-differentiated chondrosarcoma, on the other as chondroma. Since the mass showed a rather rapid growth, the diagnosis of chondrosarcoma was the most likely from the last point of view. The treatment chosen consisted of radical extirpation of the tumor performed as a large right-sided pelvic resection including the acetabulum (hemipelvectomy preserving the leg). Histopathological examination of the resected specimen demonstrated a well-differentiated chondrosarcoma.

Case 5 (K.E.) Man, 56 years of age, with low back pain and right-sided sciatica. X-ray examination revealed spotty osteolytic destructions within the proximal third in combination with periosteal thickening. The process was interpreted as chronic osteomyelitis. Some months later the patient sustained a pathological fracture through the osteolytic area in the right femur and an open biopsy was performed giving the probable diagnosis of well-differentiated chondrosarcoma. The patient was referred to the orthopedic clinic where another biopsy confirmed the diagnosis. The treatment consisted of right-sided hip disarticulation. Histopathological examination of the resected specimen confirmed the diagnosis of well-differentiated chondrosarcoma. Six months after surgery there is no evidence of recurrence or metastases.

Case 6 (A.K.) Woman, 60 years of age, with a gradually increasing palpable mass in the left lower abdomen and in the left groin. X-ray examination showed a slightly osteolytic process in the acetabulum roof with a large protrusion of tumor mass into the inferior pelvis. Attempts at needle biopsy failed to give a diagnosis, but open biopsy established the diagnosis of chondrosarcoma. Left-sided pelvic resection, including the tumor, was performed (hemipelvectomy preserving the leg of Case 4). Histopathological examination of the resected specimen demonstrated a well-differentiated chondrosarcoma. Within 6 months after surgery two local recurrences developed, one in the proximal part of the thigh, one in the symphyseal area. They were both radically removed. There are no signs of metastases.

Case 7 (V.E.) Man, 52 years of age, with a football-sized bony mass in the lateral part of the right shoulder region. X-ray examination showed a very marked osteolytic destruction of the proximal humerus and the scapula, with an abundance of calcifications in the protruding tumor mass. Osteolytic destructions, interpreted as metastases, were also noted in several ribs on the right side. A fore-quarter amputation was performed and histopathological examination of the resected specimen gave the diagnosis of a well-differentiated chondrosarcoma. Two months after surgery there are no signs of pulmonary metastases. Rib resections are planned for the lesions in these areas.

MATERIALS AND METHODS

Morphology. Small pieces (approximately $1 \times 1 \times 1$ mm) are excised from the tumor tissue and immersed in saline solutions. The primary fixative was either 2% osmium tetroxide (OsO_4) buffered with α -collidine or unbuffered 2% glutaraldehyde (GA) buffered with 0.1 M cacodylate (cac) and containing 0.1 M sucrose (scr). Fixation times were 1 hour with OsO_4 and 24 hours with GA. When GA was used as primary fixative, oxidation in OsO_4 was always performed for 1-2 hours. After dehydration and embedding in Epon 812, $\sim 1 \mu$ thick sections were prepared on glass knives, stained with alkaline uranylacetate blue and examined for orientation in the blocks. After selecting a suitable area (if the embedded material, thin sections were cut on diamond knives, and were subsequently stained with lead citrate. Examination of the material thus obtained was carried out in a Siemens Elmiskop I electron microscope or in a Jeol 100 C electron microscope.

Histochemistry. Fixation was performed by immersing tumor tissue samples for 12-24 hours in 2% GA containing 0.1 M cac and 0.1 M scr (total osmolality of the fixative ~ 510 mOsmol, pH 7.2). The tissue was subsequently transferred in a running solution composed of 0.1 M cac (pH 7.2) and 0.1 M scr. Approximately 50 μ thick sections of tissue which had been pre-treated with 10% DMSO (7) were cut on a Leitz freezing microtome. The sections were rinsed in 0.1 M cac, 0.1 M scr before incubations for the demonstration of alkaline phosphatase (5) and ATPase (6). NaF and EDTA were used as inhibitors in accordance with earlier studies (5, 6). Controls were incubated in media lacking the substrates ^{32}P - β -glycerophosphate and Na ATP respectively. After incubation, sections were rinsed in a solution containing 0.1 M cac and 0.1 M scr (pH 7.2) and were subsequently postfixed in 2% α -collidine buffered OsO_4 for 1-2 hours. The further preparation for electron microscopy is described above (cf. Morphology).

RESULTS

1 Fine Structure

The tumor tissue in the cases of chondrosarcoma was found to be composed of two basic cell types: a chondrocyte-like cells of common occurrence, and b (less frequent) large mitochondria-rich cells.

a The fine structure of the chondrocyte-like cells is illustrated in Figures 1-4. These cells are elongated with an irregular cellular outline and small blunt protrusions containing cellular organelles. The ends of the cells are often pointed, forming villous-like projections (Fig. 2). Occasional villous-like projections are also noted on other areas of the cells (Fig. 1).

The outlines of the nuclei are either smooth or irregular. In some cells deep infoldings of the nuclear membranes are noted creating images such as that presented in Figure 1 where two nuclear

portions are connected by a very thin stalk. Other cells appeared to contain two or three separate nuclei. The chromatin material was evenly dispersed and margination of chromatin was not noted. The nucleoli varied in size and shape and some were very large and irregular.

Prominent features in the cytoplasm were the extremely large Golgi areas and the abundance of glycogen in the cytoplasmic ground substance. The amount of the endoplasmic reticulum varied greatly between different cells. The cisternae were of variable width and formed an irregular network of partly interconnected profiles containing a finely granular moderately electron dense material in their lumen. There seemed to be an inverse relation between the amount of glycogen and the number of the endoplasmic reticulum cisternae. In glycogen-rich cells (Figs 1 and 2), the rough surfaced endoplasmic reticulum was located at the periphery of the cytoplasm and in areas bordering on the glycogen areas. The predominant type of endoplasmic reticulum was the rough surfaced variety. The cisternae were irregular elongated and branching with occasional dilatations. Usually the cisternae contained a finely granular material. An intimate relationship between the Golgi regions and the rough surfaced endoplasmic reticulum was also noted (Fig. 2). Mitochondria were sparse or moderately abundant, lysosomes were small and few. A characteristic feature of the cytoplasmic ground substance was the occurrence of bands of tightly packed microfilaments, usually running along and immediately below the plasma membrane (Figs 1 and 2). Fat droplets were sometimes observed in the cytoplasmic ground substance (Fig. 2). Cells rich in glycogen occasionally exhibited single membrane limited bodies containing particulate glycogen (Fig. 3), probably representing autophagic vacuoles.

b The mitochondria-rich cells were large with abundant cytoplasm and extremely irregular outlines (cf. Figures 5 and 6). These cells were often multinucleated with highly irregular nuclear membranes. Large numbers of mitochondria of variable size were present in these cells. The extension of the rough surfaced endoplasmic reticulum varied. In some areas, the endoplasmic reticulum was prominent (cf. Fig. 8) and usually formed long, slender cisternae of the rough surfaced variety. The membranes bordering these cisternae usually ran in an approximately parallel course and irregular widenings of the type observed in the chondrocyte-like cells were not observed. Golgi regions were not prominent in these cells and lysosomes were few. Particulate glycogen and fat droplets were not present in the ground cytoplasm of these cells.

ultrastructural localization of enzymes in human neoplasms appear however to indicate that the prospect for further development in this field is encouraging.

The ultrastructural localization of phosphatases, such as adenosine triphosphatase (ATPase) and nonspecific alkaline phosphatase, in normal bone and cartilage tissue appears to be rather well documented.

By application of improved methods for preparation and incubation of normal hard tissues or bone tumor samples, it should be possible to obtain valuable information regarding modifications in the histochemical pattern of these enzymes in bone tumor cells as related to the situation in normal bone and cartilage cells. Even though this approach is still in a preliminary or data-collecting phase in morphologic cancer research, one important concept has emerged namely that some neoplasms can be classified as to their cell or tissue of origin by detecting within the tumor a marker enzyme of the parent tissue.

In the present investigation, the fine structure and localization (at the electron microscope level) of alkaline phosphatase and ATPase has been studied using previously established methodology (5, 6)

CASE HISTORIES

Case 1 (U.S.) Man, 57 years of age, with gradually increasing pain in the left shoulder region. X-ray examination showed a picture typical of chondrosarcoma of the scapula, this diagnosis being confirmed by needle biopsy. Removal of the tumor was carried out by scapulectomy. Histopathological examination of the resected specimen showed a well-differentiated chondrosarcoma. Two years later there are no signs of recurrence or metastases.

Case 2 (U.E.) Man, 54 years of age, with a painless palpable mass in the distal part of the right thigh. X-ray examination demonstrated an osteolytic process in the femur extending into the soft tissue. Open biopsy gave the diagnosis of a well-differentiated chondrosarcoma. Amputation was advised, but the patient refused and disappeared.

Case 3 (A.S.) Woman, 65 years of age, with a history of low-back pain and right-sided sciatica. X-ray examination revealed an osteolytic destruction of the body of the 4th lumbar vertebra. Needle biopsy gave the diagnosis of chondrosarcoma. The patient was operated upon in two stages. At the first stage a laminectomy was carried out on the 4th lumbar vertebra, followed by posterior fixation with two Harrington rods. At the same time, tissue was curetted from the vertebral body and the diagnosis of well-differentiated chondrosarcoma was histopathologically verified. At the second-stage operation, the main part of the body of the 4th lumbar vertebra, including the tumor, was resected. The defect was filled with a block of bone-cement. More than one

year after the operation, the patient is symptom-free; there are no signs of local recurrence or metastases.

Case 4 (K.L.) Man, 19 years of age, with pain in the right groin upon sport activities. X-ray examination demonstrated a calcified mass originating from the ilio-pubic bone and extending behind the symphysis to dislocating the urinary bladder. Open biopsy was performed twice. On one occasion the histopathology of the resected tissue was interpreted as well-differentiated chondrosarcoma, on the other as chondroma. Section mass showed a rather rapid growth, the diagnosis of chondrosarcoma was the most likely from this last point of view. The treatment chosen consisted of total extirpation of the tumor, performed as a large right-side pelvic resection including the acetabulum (hemipelvectomy preserving the leg). Histopathological examination of the resected specimen demonstrated a well-differentiated chondrosarcoma.

Case 5 (N.E.) Man, 56 years of age, with low back pain and right-sided sciatica. X-ray examination revealed spotty osteolytic destructions within the proximal femur in combination with periosteal thickening. The pain was interpreted as chronic osteomyelitis. Some months later the patient sustained a pathological fracture through the osteolytic area in the right femur and an open biopsy was performed giving the preoperative diagnosis of well-differentiated chondrosarcoma. The patient was referred to the orthopedic clinic where another biopsy confirmed the diagnosis. The treatment consisted of right-sided hip disarticulation. Histopathological examination of the resected specimen confirmed the diagnosis of well-differentiated chondrosarcoma. Six months after surgery there is no evidence of recurrence or metastases.

Case 6 (A.N.) Woman, 60 years of age, with a gradually increasing palpable mass in the left lower abdomen and in the left groin. X-ray examination showed a slightly osteolytic process in the acetabulum roof with a large protrusion of tumor mass into the inferior pelvis. Attempts at needle biopsy failed to give a diagnosis, but open biopsy established the diagnosis of chondrosarcoma. Left-sided pelvic resection, including the tumor, was performed (hemipelvectomy preserving the leg, cf. Case 4). Histopathological examination of the resected specimen demonstrated a well-differentiated chondrosarcoma. Within 6 months after surgery two local recurrences developed, one in the proximal part of the thigh, one in the symphyseal area. They were both radically removed. There are no signs of metastases.

Case 7 (V.E.) Man, 52 years of age, with a football-sized bony mass in the lateral part of the right shoulder region. X-ray examination showed a very marked osteolytic destruction of the proximal humerus and the scapula, with an abundance of calcifications in the protruding tumor mass. Osteolytic destructions, interpreted as metastases, were also noted in several ribs on the right side. A fore-quarter amputation was performed and histopathological examination of the resected specimen gave the diagnosis of a well-differentiated chondrosarcoma. Two months after surgery there are no signs of pulmonary metastases. Rib resections are planned for the lesions in these areas.

MATERIALS AND METHODS

cytology Small pieces (approximately $1 \times 1 \times 1$ mm) were excised from the tumor tissue and immersed in fixative solutions. The primary fixative was either 2% osmium tetroxide (OsO_4) buffered with α -collidine or chilled 2% glutaraldehyde (GA) buffered with 0.1 M dextro cacodylate (cac) and containing 0.1 M sucrose (scr). Fixation times were 1 hour with OsO_4 and 24 hours with GA. When GA was used as primary fixative, postfixation in OsO_4 was always performed for 1-2 hours. After dehydration and embedding in Epon 812, $\sim 1 \mu$ thick sections were prepared on glass knives, stained with alkaline toluidine blue and examined for orientation in the blocks. After selecting a suitable area of the embedded material, thin sections were cut on second knives, and were subsequently stained with lead citrate. Examination of the material thus obtained was carried out in a Siemens Elmiskop I electron microscope or in a Jeol 100 C electron microscope.

Histochemistry Fixation was performed by immersing tumor tissue samples for 12-24 hours in 2% GA containing 0.1 M cac and 0.1 M scr (total osmolality of the fixative = 510 mOsmol, pH 7.2). The tissue was subsequently transferred to a rinsing solution composed of 0.1 M cac (pH 7.2) and 0.1 M scr. Approximately 50 μ thick sections of tissue which had been pre-treated with 10% DMSO (7) were cut on a Leitz freezing microtome. The sections were rinsed in 0.1 M cac, 0.1 M scr before microtomes for the demonstration of alkaline phosphatase (5) and ATPase (6). NaF and EDTA were used as inhibitors in accordance with earlier studies (5). Controls were microtomed in media lacking the substrate (Na- β -glycerophosphate and Na-ATP respectively). After incubation, sections were rinsed in a solution containing 0.1 M cac and 0.1 M scr (pH 7.2) and were subsequently postfixed in 2% α -collidine buffered OsO_4 for 1-2 hours. The further preparation for electron microscopy is described above (cf. Morphology).

RESULTS

1 Fine Structure

The tumor tissue in the cases of chondrosarcoma was found to be composed of two basic cell types: a chondrocyte-like cells of common occurrence, and b (less frequent) large mitochondria-rich cells.

a The fine structure of the chondrocyte-like cells is illustrated in Figures 1-4. These cells are elongated with an irregular cellular outline and small blunt protrusions containing cellular organelles. The ends of the cells are often pointed, forming villous-like projections (Fig. 2). Occasional villous-like projections are also noted on other areas of the cells (Fig. 1).

The outlines of the nuclei are either smooth or irregular. In some cells deep infoldings of the nuclear membranes are noted creating images such as that presented in Figure 1 where two nuclear

portions are connected by a very thin stalk. Other cells appeared to contain two or three separate nuclei. The chromatin material was evenly dispersed and margination of chromatin was not noted. The nucleoli varied in size and shape and some were very large and irregular.

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b The mitochondria-rich cells were large with abundant cytoplasm and extremely irregular outlines (cf. Figures 5 and 6). These cells were often multinucleated with highly irregular nuclear membranes. Large numbers of mitochondria of variable size were present in these cells. The extension of the rough surfaced endoplasmic reticulum varied. In some areas, the endoplasmic reticulum was prominent (cf. Fig. 8) and usually formed long, slender cisternae of the rough surfaced variety. The membranes bordering these cisternae usually ran in approximately parallel course and irregular widenings of the type observed in the chondrocyte-like cells were not observed. Golgi regions were not prominent in these cells and lysosomes were few. Particulate glycogen and fat droplets were not present in the ground cytoplasm of these cells.

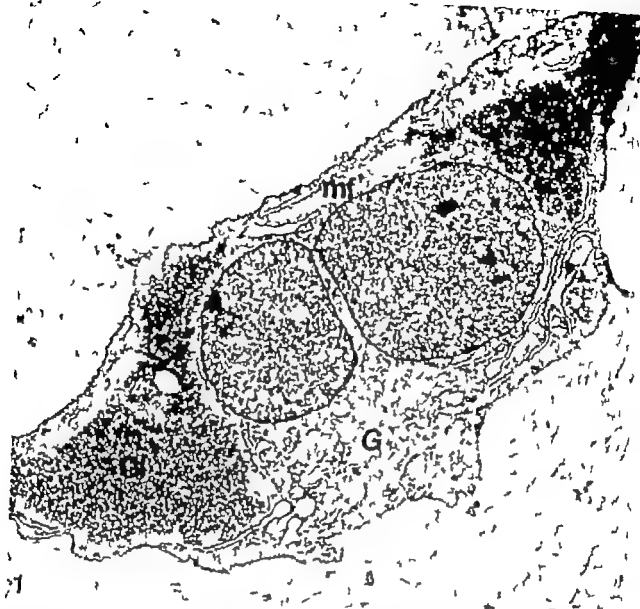


Fig 1 Survey picture of chondrocyte-like cell with highly indented nucleus. The cytoplasm contains abundant glycogen (G) and a large Golgi region (G). Some areas of the cell are crowded with microfilaments (mf). The endoplasmic reticulum is moderately well developed. Glutaraldehyde + OsO_4 , section staining with lead citrate $\times 10\,000$ Case 6

In some tumors histiocyte-like cells were also encountered.

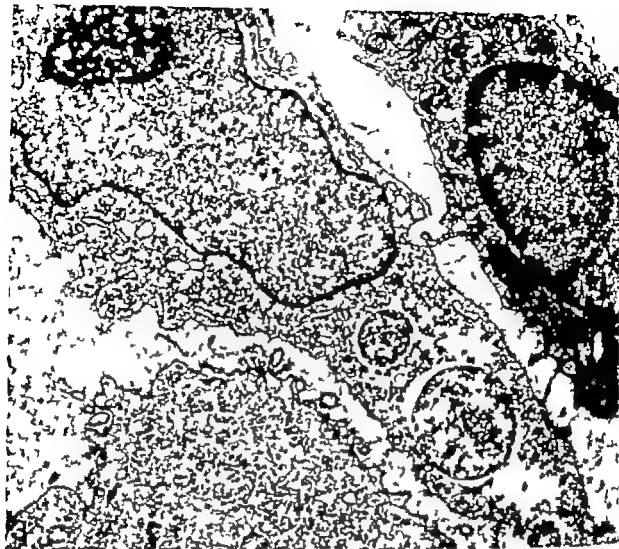
2. Localization of alkaline phosphatase

Both types of tumor cells described above showed the presence of reaction product on their plasma membranes, as illustrated in Figures 5 and 6. Reaction product was also present on infoldings of the plasma membranes and on the membranes of some vesicles and vacuoles located subjacent to the plasma membranes. Other areas of the cells were non-reactive. Reaction product was absent from cellular components in tissues incubated in a medium lacking the substrate or in a complete medium containing EDTA. A diffuse sprinkling of

lead phosphate precipitate was noted in the extracellular matrix substance in sections incubated in the complete medium as well as in medium lacking substrate or containing EDTA.

Fig 2 Portion of chondrocyte-like cell with a huge Golgi region (G), a large glycogen area (G), two fat droplets (F) and a moderately well developed, irregular rough surfaced endoplasmic reticulum concentrated to the periphery of the Golgi area and along the plasma membranes in association with bundles of densely packed microfilaments. Glutaraldehyde + OsO_4 , section staining with lead citrate $\times 10\,000$ Case 6





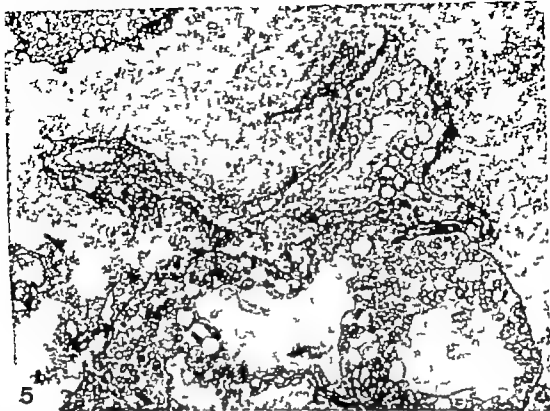




Fig 3 Portions of 3 cells are illustrated. The cell in the intermediate position is clearly of the chondrocyte-type while the one in the upper corner is probably a mitochondria-rich cell. The lower of the 3 cells contains finely distributed glycogen granules in its groundtoplasm and is possibly of the chondrocyte-like type. $\text{Histochemical: } + \text{OsO}_4$, section staining with lead citrate. $\times 9,300$, Case 3

Fig 4 Portion of a chondrocyte-like cell with immature perinuclear Golgi apparatus, L. lysosomes. $\text{Histochemical: } + \text{OsO}_4$, section staining with lead citrate. $\times 5,000$, Case 6.

Fig 5 Tissue incubated for the demonstration of alkaline phosphatase (incubation time 23 min.) Reaction product is confined to the plasma membrane of the two cells illustrated. The upper cell has a chondrocyte-like appearance while the one below contains two nuclei, abundant mitochondria and is somewhat difficult to asportize but is probably a mitochondria-rich cell. Section staining with lead citrate. $\times 4,000$, Case 3

Fig 6 Treatment as in Fig 5. A large mitochondria-rich cell with two nuclei shows presence of reaction product on its plasma membrane. Reaction product is also occurring on the plasma membrane of the small chondrocyte-like cell (in the left upper corner). Section staining with lead citrate. $\times 4,300$, Case 3

Fig 7 Tissue incubated for the demonstration of adenosine triphosphatase, incubation time 20 min. Portions of two cells are illustrated. The upper one is chondrocyte-like with reaction product on the plasma membrane and on vacuoles and vesicles in the cytoplasm; are probably representing invaginations of the plasma membrane. In the cell in the lower part of the picture, reaction product is confined to the plasma membrane. This cell corresponds to the large mitochondria-rich one illustrated in Fig 6. Section staining with lead citrate. $\times 14,000$, Case 3

Fig 8 Same treatment as in Fig 7. Portions of 3 cells are present in this picture, two chondrocyte-like ones and one large mitochondria-rich cell (to the left). Reaction product is located over the plasma membrane in all the cells. Section staining with lead citrate. $\times 13,200$, Case 3

3. Localization of ATPase

The results are illustrated in Figures 7 and 8. The localization was similar to that described for nonspecific alkaline phosphatase. In addition, reaction product was prominent on the membranes bordering capillaries. The appearance in control sections was the same as described for tissues incubated for the demonstration of alkaline phosphatase.

Chondrosarcoma of bone forms a heterogeneous group of malignant neoplasms ranging from well differentiated cases with borderline histologic malignancy to highly malignant ones with a marked tendency to metastasize. The majority of the chondrosarcomas, however, are comparatively well differentiated, yet easily diagnosed as malignant (2). All the tumors included in the present report fall into this latter category and variants such as mesenchymal chondrosarcoma (4, 15), dedifferentiated cartilaginous tumors (8), and chondrosarcomas with atypical localization have been excluded from the material studied.

Normal chondrocytes - for instance in articular cartilage - are characterized by an abundance of orderly arranged rough surfaced endoplasmic reticulum, occurrence of particulate glycogen and lipid droplets in the cytoplasmic ground substance, comparatively few mitochondria, a somewhat irregular shape of the cells, and nuclei with smooth outline. The appearance of the normal chondrocytes varies with localization within the tissue, and differences in the descriptions are noted between authors who have described these cells (10, 11, 13, 16).

Our study revealed the occurrence of two distinct types of cells in the tumor tissue. One type clearly resembled a normal chondrocyte and accordingly was termed "chondrocyte-like". These cells evidently correspond to the bulk of tumor cells described by light microscopists in studies of conventional chondrosarcomas. Differences between normal chondrocytes and the tumor cells include: (a) greater variability in shape and size of nuclei with occurrence of binucleate forms in the tumor cells, (b) more numerous and irregular protrusions of the plasma membrane in the latter, (c) a higher variability in the appearance of the rough surfaced endoplasmic reticulum in the tumors, and (d) more abundant filaments, particulate glycogen, and lipid droplets in the latter. These observations agree largely with previous descriptions of chondrosarcomas (1, 3, 4, 8, 12, 14, 15).

Occurrence of large, mitochondria-rich cells in well-differentiated chondrosarcomas has not been described previously. These cells could - on the basis of their fine structure - easily be separated from the chondrocyte-like cells, and apparent transitional forms were not encountered. The major distinguishing characteristics of these cells were - in addition to large numbers of mitochondria - their large size, irregular nuclear contours and lack of glycogen and fat droplets in the matrix. The findings indicated that the mitochondria-rich cells were prone to attain multinucleated forms, although

indications as to whether this occurred by fusion of mononuclear cells or by amitotic division of nuclei are lacking.

It is well known from light microscopic observations that the type of chondrosarcoma studied in the present report often contains multinucleated (usually binucleated) cells. The findings indicate that such multinucleated cells may correspond both to chondrocyte-like cells and mitochondria-rich ones. The latter tend to be larger than the chondrocyte-like cells and also often to contain more than two nuclei. Thus it is appropriate to refer to them as giant cells.

Multinucleated giant cells are characteristic features of a usually benign chondromatous tumor - the chondroblastoma. Levine and Bensch (9) have described the salient features of this tumor and conclude that there may be two histogenetically separate types of giant cells in these tumors - osteoclast-like ones and multinucleated tumor cells. The large multinucleated cells in the chondrosarcomas studied by us were smaller than osteoclasts, contained fewer nuclei, lacked a distinct brush border and were devoid of a lysosomal apparatus resembling that in osteoclasts. Furthermore, it appeared that the "giant cells" in the chondrosarcomas were closely related to mononuclear mitochondria-rich cells. The latter had small and few lysosomes and there was no indication that they represented macrophages or monocytes. Such cells are known to be precursors of osteoclasts. Thus, we conclude that the large multinucleated cells in the chondrosarcomas probably did not represent osteoclasts but rather true tumor cells. Neither the mitochondria-rich cells nor the large multinucleated cells bear any striking resemblance to the chondroblasts described in chondroblastomas or to other cells observed in chondrosarcoma variants* (2).

Cues to the histogenesis of different tumor cells can sometimes be obtained from histochemical studies. Characteristic localizations of ATPase and nonspecific alkaline phosphatase are noted in various types of bone cells (5, 6). Thus, alkaline phosphatase has been demonstrated to be present over the plasma membrane of chondroblasts and chondrocytes. In this respect there is no difference between the neoplastic cells in the chondrosarcomas and the normal cells. Furthermore, the two types of tumor cells appear to be similar with regard to the localization of the enzyme, although there may be quantitative differences.

Table I summarizes current knowledge concerning the localization of ATPase in different types of cells present in bone and includes the results of the present study concerning the chondrocyte-like cells and the mitochondria-rich cells. Neither normal

TABLE I Comparison of the Fine Structural Localization of Mg^{++} ATPase in Different Types of Normal or Neoplastic Cells in Calcified Tissue

Type of cell	Plasma membrane activity	Lysosomal activity
<i>Non neoplastic</i>		
Chondroblast, chondrocyte	-	+
Fibroblast, preosteoblast	+	+
Osteoblast	++	+
Monocyte, histiocyte, macrophage	+	+
Osteoclast	+	(in brush border)
<i>Chondrosarcoma</i>		
Chondroblast-like	+	
Mitochondria-rich	+	
<i>Giant cell tumor of bone†</i>		
Giant cell	-	+
Stromal cells, types 1 and 2	-	

† (unpublished results)

chondroblasts nor chondrocytes show the presence of ATPase in association with the plasma membrane. However, immature matrix-forming cells (fibroblasts, preosteoblasts) show some activity at this location while there are indications of abundant ATPase in the plasma membrane of osteoblasts (6). Both the chondrocyte-like and the mitochondria-rich tumor cells were found to have an ATPase associated with their plasma membrane and in this respect bore a resemblance to immature non-chondroid matrix-forming cells.

The results of the histochemical studies suggest that the chondrocyte-like cells have remained in an immature state with regard to ATPase. The similarity in localization of ATPase and alkaline phosphatase in the two ultrastructural types of tumor cells in the chondrosarcoma appears to support the notion that these cell types - despite their morphologic differences - still may be of similar origin. All the cell types observed in the chondrosarcomas may therefore represent morphological variants of chondroid cells.

This investigation has been aided by grants from the Swedish Cancer Society (projects number 617 B76-653 and 617 B77-06A).

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ABSENCE OF ASSOCIATION BETWEEN OESTROGEN RECEPTOR CONTENT AND IN VITRO OESTROGEN SENSITIVITY IN HUMAN BREAST CANCER

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Purpose: Skovgaard, H. Absence of association between oestrogen-receptor content and in vitro oestrogen sensitivity in human breast cancer. *Acta path. microbiol. scand. Sect. A, 86 169-172 1978*

Breast-cancer tissue from 25 consecutive patients was investigated for oestrogen receptor content and for oestrogen-induced changes in tritiated thymidine uptake by explanted cells. A higher oestrogen receptor content was not associated with any statistically significant increase in oestrogen sensitivity. Neither the dissociation constants (K_d values), nor the oestrogen binding capacity (if not oestrogen binding superinduced protein) was found to be associated with in vitro oestrogen sensitivity. One receptor-negative tumour showed oestrogen sensitivity in vitro.

Key words: Breast cancer tissue; oestrogen sensitivity in vitro; oestrogen receptors.

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Received 10 x 77 Accepted 26 x 77

It is generally assumed that the interaction between oestrogen and oestrogen-responsive cells is transmitted by an intracellular cytoplasmatic oestrogen-binding protein, an oestrogen receptor.

It has recently been shown that some female breast-cancer tissue contains oestrogen receptors, and that such tissue is more responsive to hormonal therapy than the corresponding receptor-negative tissue (e.g. McGuire (6)). Although this association is not without exceptions, the investigations clearly indicate that the presence of oestrogen receptors is essential to the control of tumour-cell proliferation in vivo.

In vitro, Lippman & Boies (4) have shown that an oestrogen-responsive human breast-cancer cell line also contained oestrogen receptors, but it has never been investigated in large series whether it can be assumed that the effect of oestrogen on in vitro proliferation is mediated by oestrogen receptors.

In order to investigate the in vitro relationship between these two parameters, the oestrogen-receptor content was determined in a portion of human breast-cancer biopsy specimens. Another portion of the same specimens was short-term cultivated, and the influence of oestrogen on tritiated thymidine (3H-TdR) uptake was determined.

MATERIALS AND METHODS

Biopsy Specimens

Breast-cancer tissue from 25 consecutive patients was investigated. Each biopsy was divided into two portions. One was tested for oestrogen sensitivity after 24 hours of in vitro cultivation. The second portion was investigated for its oestrogen-receptor content.

Culture Procedure

The method applied is described elsewhere. As it has been shown that the hormonal effect on 3H-TdR uptake in vitro can be determined as early as 24 hours after

explantation (8) the tumour biopsies were cultivated for this period before the effect of 17 beta-oestradiol (10^{-6} M) on 3H TdR uptake was tested.

Briefly small tissue fragments (approx. 0.5 mm³) were cultivated in 10 ml test tubes containing MEM supplemented with foetal calf serum to a concentration of 5 per cent in an atmosphere of 95 per cent humidified air and 5 per cent CO₂. 17-beta-oestradiol was added to some cultures when they were set up and 24 hours after explantation 3H thymidine was added in the cultures for one hour.

The results were expressed in terms of counts per minute per mg protein (CPM/mg protein). As the results may not be normally distributed, the mean values of controls and hormone treated cultures were compared by means of the non-parametric Mann-Whitney test.

Oestrogen Receptor Assay

The tissue was minced with a pair of scissors, cooled in liquid nitrogen, and homogenized in a Schwisgmühle (Reich West Germany). The homogenate was weighed and suspended in a threefold volume of TE buffer (Tris 10 mM EDTA 1.5 mM NaCl 10 mM pH 7.4). After centrifugation of the homogenate at 100 000 g and 4 °C (Beckman Spinco ultracentrifuge L50) for 1 hour the cytosol was assayed for the amount of high affinity receptors by the method of Mester *et al* (7) Fekety *et al* (3) and modified by Daehrfeldt (1).

Briefly the supernatants were incubated at 0° C with five concentrations of 3H 17-beta-oestradiol ranging from 7.2×10^{-9} M to 2.9×10^{-10} M for 2 hours (in duplicates). The incubation with 3H 17-beta-oestradiol was terminated by adding dextran-coated charcoal suspension. After adsorption for 30 minutes at 0° C the charcoal was spun down and 200 µl of the supernatant was counted for radioactivity by liquid scintillation. Quench correction was performed by the channel ratio method. To correct for deficient adsorption controls were used containing 3H 17-beta-oestradiol, TKE buffer and bovine albumin. The counts from these were subtracted from experimental values. The binding capacities were read from a Scatchard plot which makes possible a correction of unspecific binding of 3H 17-beta-oestradiol as well as a determination of K_d values (dissociation constants). When the results were scattered along a straight line with no slope (unspecific binding) or no significant binding was recorded the tumours were defined as receptor negative and tumours were defined as receptor positive when the binding results were scattered along a sloping straight line. Initial studies (Fajusen unpublished) showed that the binding capacities and the K_d values could be estimated with an accuracy of 10–12%.

Tissue Distribution of Oestrogen Receptors

As already mentioned, one portion of each tumour biopsy specimen was investigated for oestrogen receptor content and another for oestrogen influence on the in vitro 3H TdR uptake. This approach presupposes that the oestrogen receptors are distributed in a relatively even fashion throughout the tumour biopsy. In order to

TABLE 1 Comparison of the Oestrogen Receptor (ER) of two Portions of the Same Tumour in 5 Cancer Breast Tumours

Tumour No	Portion No. 1	Portion No. 2
	f mol oestrogen bound/mg protein	f mol oestrogen bound/mg protein
1	0	0
2	0	0
3	49	54
4	0	0
5	0	0

f mol = 10^{-15} mol.

test this assumption, each of five tumours were cut into two portions, and the oestrogen receptor was assayed in each portion. From Table 1 it appears that the two portions were comparable as regards oestrogen receptor content. Consequently it was justifiable to use one portion of a tumour biopsy for oestrogen-receptor determination and the other portion for oestrogen sensitivity in vitro.

RESULTS

From Table 2 it appears that breast-cancer tissue from 9 out of 20 patients contained oestrogen receptors. Four of these were oestrogen-sensitive in vitro. One receptor-negative tumour also showed oestrogen sensitivity in vitro. The K_d values (dissociation constants) varied from 10^{-9} M to 10^{-11} M which means that the binding protein (oestrogen receptor) has a very strong affinity for oestrogen. Further as appears from Table 2 there is no significant difference in the K_d values between oestrogen-sensitive and non-sensitive tumours. Finally no relation was demonstrated between binding capacity (expressed as f mol bound oestrogen/mg supernatant protein) and the oestrogen sensitivity.

When tested with Fisher's exact test, statistical evidence could not be demonstrated between presence of oestrogen receptors and in vitro oestrogen sensitivity ($P > 0.10$).

DISCUSSION

A number of objections may be raised against the method applied in the present study.

1. As discussed elsewhere (8) the in vitro DNA synthesizing cells may not be representative of the whole tumour-cell proliferation. In order to minimize the possibility of selective cell proliferation in culture period was restricted to 24 hours.

TABLE 2 The Oestrogen Receptor Content (ER) in Relation to Oestrogen Sensitivity *in vitro* in 20 Comedocarcinoma Breast Tumours

Patient No	ER [fmol oestrogen bound/ mg prot	K _d M	In vitro sensitivity
1	43	2×10^{-9}	Positive
2	0	—	Negative
3	0	—	Negative
4	0	—	Negative
5	125	2×10^{-10}	Negative
6	0	—	Negative
7	0	—	Positive
8	0	—	Negative
9	0	—	Negative
10	11	7×10^{-10}	Negative
11	35	6×10^{-11}	Negative
12	56	1×10^{-11}	Positive
13	0	—	Negative
14	80	2×10^{-10}	Negative
15	43	0.7×10^{-11}	Positive
16	0	—	Negative
17	22	1×10^{-11}	Positive
18	0	—	Negative
19	0	—	Negative
20	49	4×10^{-11}	Negative

[fmol 10^{-15} mol

K_d the dissociation constants

Positive significant difference in 3H-thymidine uptake in hormone treated cultures compared to controls ($P < 0.05$)

Negative no significant difference in 3H-thymidine uptake in hormone treated cultures compared to controls ($P > 0.05$)

2 It is conceivable that oestrogen receptors are not from the cells *in vitro*. However, Lippman *et al* (4, 5) have shown that hormone receptors are preserved even in long-term cultures.

3 The possibility that the oestrogen receptor content is unevenly distributed in different parts of the same tumour is contradicted by the observations presented in Table 1.

4 Concerning the presence of endogenous oestrogen may suppress the activity of hormone added to the culture medium. However in the present investigation, 9 tumours contained receptors and consequently took up added oestrogen (Table 2). The fact that 5 of these 9 tumours were insensitive to experimentally added oestrogen shows that this lack of sensitivity cannot be due to a blocking effect exerted by endogenous oestrogen.

Furthermore, one tumour showed *in vitro* oestrogen sensitivity in spite of the fact that no oestrogen receptors could be demonstrated. As the

patient was 20 years past menopause and had received no hormonal therapy before the investigation, it seems unlikely that the negativity of the receptors could be due to the receptors being blocked by endogenous hormone.

5 One or more unknown factors may co-operate with oestrogen in *in vitro* proliferation regulation. In experimentally induced hormone-dependent mammary tumours Doo & Sunka (2) have shown that the presence of several hormones is a precondition for *in vitro* DNA synthesis. It is possible that endogenous content of such hormones and perhaps some unknown factors may explain why some receptor-positive tumours were sensitive to oestrogen and others were not.

In conclusion, the present investigation suggests that *in vitro* oestrogen sensitivity is not associated with the presence of oestrogen receptors. This conclusion must of course be evaluated in the light of the limited experimental material. Further studies

are clearly needed before the role of oestrogen receptors in the *in vitro* proliferation of breast cancer tissue can be definitely determined. Of central importance for this problem is the question whether oestrogen receptors are specific in all human breast tumours. Current experiments in our laboratory are focused on this question.

The author wishes to thank Drs. *P. Frederiksen* and *E. Sommer Hansen* the University Institute of Pathology Aarhus Kommunehospital for the histological examinations, Professor *S. Kaag* the Radium Centre, Aarhus Kommunehospital and Dr. *P. Linnet Jepsen* the Department of Surgery I Aarhus County Hospital, for helpful discussions in preparing the manuscript, and Mrs. *Mette Juhl* and Mrs. *Karen Thomsen* for skilful technical assistance.

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HUMAN MALIGNANT LYMPHOMAS IN VITRO

Characterization of Biopsy Cells and Establishment of Permanent Cell Lines

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Sundstrom, C. & Nilsson, K. Human malignant lymphomas in vitro. Acta pathol. microbiol. scand. Sect. A, 86: 173-184, 1978.

A series of 111 biopsies from different types of malignant lymphomas were characterized in short-term culture experiments and during prolonged growth *in vitro*. The majority of the lymphocytic lymphomas and half of the histiocytic lymphomas expressed surface immunoglobulins, either in monoclonal or polyclonal form, indicating B-lymphocyte derivation. No lyszyme production was noted in either type of lymphoma, giving further support to the notion that histiocytic lymphomas are not truly histiocytic. Production of β_2 -microglobulin was higher in histiocytic than in lymphocytic lymphomas and Hodgkin's disease but did not significantly differ from the production observed in non-neoplastic lymph node disorders. Incorporation of 3 H-thymidine varied greatly within each category of lymphoma, the highest mean labeling index was noted in histiocytic lymphomas, possibly reflecting the generally more malignant course in such cases. Epstein-Barr virus-associated nuclear antigen was observed in one case of Hodgkin's disease. Attempts to establish permanent tumor cell lines were successful only from two explants of lymphocytic lymphoma and one pleural effusion from histiocytic lymphoma. The two cell lines derived from lymphocytic lymphomas both exhibited B-lymphocyte characteristics. The histiocytic lymphoma line lacked lymphocyte markers, produced lyszyme and was found to be rich in cytoplasmic esterase. These features are consistent with a true histiocytic derivation of this line. Lymphoblastoid cell lines representing non-neoplastic EBV-carrying lymphocytes contaminating the biopsies were derived from 19 biopsies, with the highest frequency noted in cultures of biopsies from Hodgkin's disease. The tumor lines were all EBV-genome negative.

Key words: Lymphomas, human malignant, cell lines.

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Received 4 vii 77. Accepted 31 x 77.

The biology *in vitro* of non-Burkitt lymphomas has been the subject of several reports previously. Some of these have dealt with surface characteristics, i.e. B- and T-lymphocyte markers of fresh biopsy cells in short-term culture (1, 5, 6, 9, 19, 23, 30, 31, 34, 47, 78). Others concerned the establishment and characterization of permanent lymphoid cell lines (7, 8, 12, 13, 18, 32, 35, 50, 55, 56, 60, 61, 65, 68, 79, 80, 81). Attempts to clarify the etiology of non-Burkitt lymphomas have concentrated on the search for Epstein-Barr virus (EBV) (24, 25, 40, 64, 73).

It can be concluded from the above studies that: a) lymphocytic lymphoma (LL) is a homogeneous group with respect to the *in vitro* features. Virtually all biopsies have shown B-cell characteristics. Histiocytic lymphoma (HL) has, on the other hand, been heterogeneous. Some tumors have behaved as B-cell neoplasms while others have displayed classical histiocytic characteristics. b) attempts to establish permanent lines of cells representing the tumor cell population in the biopsy have mostly failed, but notably the frequency of EBV-carrying non-neoplastic B-lymphoblastoid cell lines (LCL) has been high (11, 56, 63, 81); so far only two LL

and four HL lines have been documented (13, 61, 79): c) EBV does not seem to be associated with non-Burkitt lymphomas. In no instance has a tumor been found to carry Epstein-Barr nuclear antigen (EBNA) or EBV genome as examined by sensitive nucleic acid hybridization technique (for a review, see Klein 41); d) no convincing evidence of a C-type viral etiology been presented although «footprints» of putative RNA viruses have been claimed to be present in several lymphoma biopsies by the Spiegelman group.

In this study we report our experience with biopsy cells in short and long-term *in vitro* culture from histologically different types of lymphomas obtained from a series of 55 unselected patients. The cells were characterized in short term culture with regard to morphology, growth, ^3H -thymidine incorporation, presence of EBNA and production of immunoglobulin, β_2 -microglobulin and lysozyme. Long term cultures were initiated with the aim of establishing permanent cell lines. This paper contains a description of the 22 derived lines, with special reference to the question of whether they originated from tumor cells or non-tumor cells present in the explants.

MATERIAL AND METHODS

Tumor Material

Forty-five surgical biopsies and 10 pleural or peritoneal effusions from malignant lymphomas were examined. The biopsy material comprised 38 tumorous lymph nodes, three spleens, two tonsils and tumor infiltrations in one thyroid and one submandibular gland. The histo-pathological classification was made independently by the authors and at least one other pathologist, according to Rappaport (71) and Lukes et al. (51). The biopsies were transported to the laboratory in culture medium. Tumor cell effusions were transported without further treatment. Biopsies were scalped into pieces and the cut surfaces scraped to obtain a tumor cell suspension in culture medium. Parts of the biopsies were cut into approx. 1 mm³ pieces for use in Spongostan grid cultures (see below). Cells in effusions were harvested by sedimentation (1 \times G) or centrifugation (400 \times G 10 min). The cell pellet was then suspended in culture medium. Effusions containing a significant amount of erythrocytes were centrifuged on a Ficoll-Isopaque gradient according to Boyum (4). Finally the viability of the suspended tumor cells was estimated with the trypan blue exclusion method.

Cell Surface Immunoglobulin

Surface localized immunoglobulin (S-Ig) was determined by indirect immunofluorescence as detailed (61, 79).

Production of β_2 -microglobulin

Production of β_2 -microglobulin (β_2 - μ) was quantitated by radioimmunoassay (16). Briefly, 10⁶ cells were

washed in medium and incubated for 48 h in 2 ml of medium containing calf serum and antibiotics. The cells were then pelleted by low-speed centrifugation and the supernatant analyzed for β_2 - μ .

Production of Lysozyme

Lysozyme production was quantitated as described (75) by the *Micrococcus* lysostaphin technique (66). Es. lysozyme (Sigma Chemicals, St. Louis, Mo. USA) was used as standard. Concentrated supernatants for producing and non-producing established cell lines (79) were used as controls.

DNA Synthesis

^3H -thymidine labeling. 2×10^6 living cells in 10 ml of F 10 medium supplemented with 10% newborn calf serum were incubated for 60 min at +37°C in humidified 5% CO₂-10 air atmosphere. Then 8 ml of PBS containing 2 μCi of ^3H -thymidine (specific activity 1 Ci/ μmol) (Radiochemical Centre, Amsterdam, Expts.) was added and the cultures were incubated for another 60 min at +37°C. Labeling index (LI) was determined by autoradiography. In each test, cells harvested from logarithmically growing hematopoietic cell lines (U-267 Bm, U-698 M, U-937 K, 562, CCRF-CEM) were included as references. In these lines LI ranged between 22–58%.

Autoradiography. Cells were smeared onto microscope slides in a cytocentrifuge (Shandon Scientific Comp. Ltd., London). Air-dried slides were then washed in PBS and fixed in methanol-acetic acid (3:1) and autoradiograms prepared as described (59). Two thousand cells were counted to determine the labeling index (LI).

Non-neoplastic controls. Non-neoplastic lymphoid cells from five different sources were studied: peripheral blood lymphocytes from healthy blood donors (1) and from patients with heterophile antibody positive atypical mononucleosis (2); cells from a normal lymph node (3) and from nodes with non-specific lymphadenitis (4) and, finally lymphocytes from two normal thyroids (5).

Epstein Barr Nuclear Antigen

EBV associated complement fixing nuclear antigen (EBNA) was tested according to Reedman and Klein (72). Reagents were kindly provided by Dr. George Klein, Stockholm. Each test included one negative and two positive control sera. Raji cells (69) and U-698 cells (42, 61) were used as positive and negative controls, respectively.

Culture Methods

Grid cultures. Solid tumor pieces (approx. 1 mm³) were incubated on top of Spongostan (Ferrostan, Malmo, Sweden) grids as described earlier (58). When an effusion was the starting material, cells were harvested by centrifugation, resuspended in 0.2–0.5 ml of F 10 medium, and pipetted into Spongostan gelatin foam on top of the stainless steel grids. The grids were incubated in a humidified 5% CO₂-10 air atmosphere at +37°C.

a 60 mm Nuncion Petri dishes (Nunc, Roskilde, Denmark). From each biopsy a maximum of four grid squares was initiated. Half of the cultures contained a monolayer of adult allogeneic skin fibroblasts or fat cells. The culture medium, Ham's F-10 (Ham, MD), supplemented with 10% newborn calf serum (NCS) and antibiotics (100 IU-penicillin/ml, 50 µg streptomycin/ml, 1.25 µg amphotericin B/ml) was changed twice weekly. At medium renewal the spent medium, containing the cells discharged from the grids, was transferred to 50 ml Erlenmeyer flasks containing a monolayer of the above types of feeder cells. One third of the medium in the flasks was renewed twice weekly.

The morphology of tumor cells, their growth patterns in suspension and their relation to feeder cells were studied at regular intervals in an inverted microscope (Leitz, Wetzlar, Germany). The appearance of cells other than tumor cells was also noted. Each culture was below used until a permanent cell line had been established or until the production of cells had deteriorated completely (a maximum of eight weeks).

Growth Curves

The primary growth rate of the tumor cell suspension was studied in selected cases. Total cell number was estimated by a Celloscope 502 (AB Lars Ljungberg, Stockholm, Sweden) and viability by the trypan blue exclusion method. Several parallel Erlenmeyer flask cultures were initiated in each case (10^5 cells/ml in 10 ml) with and without feeder cells (glial cells). The medium (Ham's F-10) was supplemented with 10% NCS or 10% fetal calf serum. The labelling index was determined in these cases as described above.

RESULTS

Morphological Classification

The histopathological diagnosis and sex distribution in the 55 tumors are summarized in Table 1. Twenty nine LL, 11 HL and 15 cases of Hodgkin's disease (HD) were investigated. The LL and HL were mostly of the diffuse, poorly-differentiated type, 23 and 10 cases, respectively. In the HD cases the mixed type predominated.

Functional Characteristics of Biopsy Cells

Surface immunoglobulin (S-Ig) (Table 2). S-Ig was detectable in 11/18 LL biopsies. In five of these cases the S-Ig profile indicated a monoclonal tumor while in the other cases a bi- or polyclonal pattern was found. Out of eight HL four were S-Ig positive. Two of these had a monoclonal pattern. In case 878 almost all cells (95–100%) were stained by anti γ μ and 37% by anti- λ . In a fifth case (1077) only a fraction of the cells (30%) expressed light chains of γ -type. A few HD cases were investigated. Owing to the extensive contamination of reactive cells in these cases the polyclonal S-Ig pattern was not conclusive.

Production of β_2 -microglobulin (Fig. 1). Compared to the production by normal lymph node and tonsil cells (62) the LL (mean 54, range 6–153 μ g/ 5×10^5 cells/48 h) and HD biopsies (mean 86, range 12–202 μ g/ 5×10^5 cells/48 h) produced equivalent amounts of β_2 - μ . The six HL biopsies produced

TABLE 1 Total Material of Investigated malignant L. lymphomas and Mean Viability in Tumor Cell Suspensions

Diagnosis	No. of biopsies or effusions	Males	Females	Viability (mean)	Viability (range)
Lymphocytic lymphomas	29	13	16	92.3%	(65–100)
Diffuse, well differentiated	2	2	0		
Diffuse, poorly differentiated	23	10	13		
Nodular, poorly differentiated	4	1	3		
Histiocytic lymphomas	11	9	2	86.0%	(67–99)
Diffuse	10	8	2		
Nodular	1	1	0		
Hodgkin's disease	15	11	4	85.5%	(60–100)
Lymphocytic predominance		1	1		
Nodular sclerosing	3	2	1		
Mixed cellularity	8	6	2		
Lymphocytic		2	0		
Total	55	33	22		

and four HL lines have been documented (13, 61, 79). c) EBV does not seem to be associated with non Burkitt lymphomas, in no instance has a tumor been found to carry Epstein Barr nuclear antigen (EBNA) or EBV genome as examined by sensitive nucleic acid hybridization technique (for a review see Klein 41). d) no convincing evidence of a C-type viral etiology been presented although «footprints» of putative RNA viruses have been claimed to be present in several lymphoma biopsies by the Spiegelman group.

In this study we report our experience with biopsy cells in short and long term *in vitro* culture from histologically different types of lymphomas obtained from a series of 55 unselected patients. The cells were characterized in short term culture with regard to morphology, growth, ^3H -thymidine incorporation, presence of EBNA and production of immunoglobulin, β_2 -microglobulin and lysozyme. Long term cultures were initiated with the aim of establishing permanent cell lines. This paper contains a description of the 22 derived lines, with special reference to the question of whether they originated from tumor cells or non tumor cells present in the explants.

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washed in medium and incubated for 48 h in 2 ml of medium containing calf serum and antibiotics. The cells were then pelleted by low-speed centrifugation and the supernatant analyzed for β_2 -u.

Production of Lysozyme

Lysozyme production was quantitated as detailed (7) by the *Micrococcus lysostye* technique (60). Ig lysozyme (Sigma Chemicals, St. Louis, Mo, USA) was used as standard. Concentrated supernatants from producing and non-producing established cell lines (79) were used as controls.

DNA Synthesis

^3H thymidine labelling. 2 \times 10⁶ living cells in 1 ml of F 10 medium supplemented with 10% newborn calf serum were incubated for 60 min at +37°C in a humidified 5% CO₂-in-air-atmosphere. Then 0.2 ml PBS containing 2 μCi of ^3H -thymidine (specific activity 1 Ci/mmol) (Radiochemical Centre, Amersham, England) was added and the cultures were incubated for another 60 min at +37°C. Labelling index (LI) was determined by autoradiography. In each test, cells harvested from logarithmically growing hematopoietic cell lines (U-25, Bm U-698, M U-937, A 562, CCRF-CEM) were included as references. In these lines LI ranged between 22–58%.

Autoradiography. Cells were smeared onto microscope slides in a cytocentrifuge (Shandon Scientific Comp. Ltd., London). Air-dried slides were then washed in PBS and fixed in methanol/acetic acid (3/1) and autoradiograms prepared as described (59). Two thousand cells were counted to determine the labelling index (LI).

Non neoplastic controls. Non-neoplastic lymphoid cells from five different sources were studied: peripheral blood lymphocytes from healthy blood donors (1) and from patients with heterophile antibody positive infectious mononucleosis (2), cell from a normal lymph node (3) and from nodes with non-specific lymphadenitis (4) and, finally lymphocytes from two normal thyroids (5).

Epstein Barr Nuclear Antigen

EBV-associated complement fixing nuclear antigen (EBNA) was tested according to Reedman and Klein (72). Reagents were kindly provided by Dr. George Klein, Stockholm. Each test included one negative and two positive control sera. Raji cells (69) and U-698 cells (42, 61) were used as positive and negative controls, respectively.

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Grid cultures. Solid tumor pieces (approx. 1 mm³) were incubated on top of Spongostan (Ferrosan, Malmö, Sweden) grids as described earlier (58). When an effusion was the starting material cells were harvested by centrifugation, re-suspended in 0.2–0.5 ml of F 10 medium, and pipetted into Spongostan gelatin foam on top of the stainless steel grids. The grids were incubated in a humidified 5% CO₂-in-air atmosphere at +37°C.

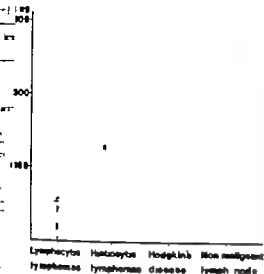


Fig 1 Production of β_2 -microglobulin (log/5 $\times 10^5$ cells \times 48 h) by different lymphomas and control lymph nodes

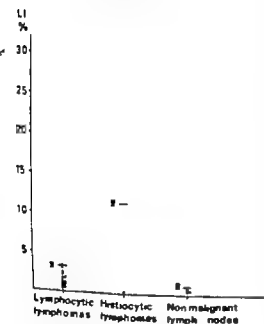


Fig 2 Labeling index in different lymphomas and control lymph nodes

differentiated lymphomas varied between 1.2 and 2.2%. Of the five examined *HL* three exhibited a high LI (31.0, 14.7 and 9.1%), while two cases gave lower values (7.2 and 0.2%). Of the control cells normal peripheral blood lymphocytes gave LIs ranging from 0.1–0.3%. The LIs in patients with infectious mononucleosis ranged from 0.1–3.3%.

The cells from a normal lymph node had the LI 0.5%. Two normal thymuses gave the LIs 2.4 and 7.5%. Four lymph nodes with non-specific lymphadenitis showed LIs ranging between 0.8–2.8%.

EBNA The presence of EBNA was tested in the tumor-cell suspension of 33 lymphomas (23 LL, 7 HL and 3 HD). All cases except one, an HD type lymphocytic depletion, were negative. In the exceptional tumor the positive cells were relatively large-sized, but whether they were tumor cells or represented B-lymphoblastoid cells contaminating the tumor could not be determined. Five control lymph nodes with non-specific lymphadenitis were all negative.

In vitro Cultivation

Initial cell viability When the tumor-cell suspension had been prepared the cell viability varied greatly (60–100%) between the tumors. The mean initial cell viability for the different types of lymphomas is given in Table 1. No significant differences were observed between the well-differentiated and the poorly-differentiated LL and HL cases. In the cases of HD where a low viability was found, a selective death of large cells was noted.

***In vitro* characteristics** The production of cells from the grids varied considerably among the biopsies, depending on such factors as initial cell viability, amount of tissue explanted and serum batch in use.

Explanted diffuse LL of both well and poor differentiation generally yielded a homogeneous population of round, mostly non-clumping cells, which attached loosely to the feeder cells. The tumor-cell size varied somewhat with the morphological «differentiation». A few non-neoplastic lymphocytes were seen, which often survived longer than the tumor cells. A more heterogeneous cell population was found in nodular LL. The HL generally dislodged large round tumor cells from the grids, which like poorly-differentiated LL did not clump and adhered loosely to feeder cells. In a few cases, however (e.g. biopsy 937) the tumor cells varied somewhat in shape. Round cells were mixed with ovoid-polygonal cells with short cytoplasmic projections. These histiocyte-like cells did not clump but adhered sometimes to glass and plastic surfaces. The tumor cells in LL and HL survived for a few (2–4) weeks, after which time only non-neoplastic lymphocytes remained in the cultures. The HD biopsies produced a highly heterogeneous cell population from the grids. This population of cells mostly consisted of non-neoplastic lymphocytes of varying shape, often clumping and adhering to the feeder cells. Some large cells were binucleated and resembled Reed-Sternberg cells. They adhered to

TABLE 2 Surface Immunoglobulin Pattern in Malignant Lymphoma Biopsy Cells

Diagnosis ¹ Biopsy or effusion No	Poly	α	δ	γ	μ	λ	Interpretation
Lymphoma in lymph nodes							
875 DPD	nt	nt	nt	15	27	0	15 Tumor cells probably IgM
882 DPD	nt	nt	nt	54	64	68	45 Poly clonal pattern
892 DWD	nt	0	nt	11	3	0	11 Tumor cells probably S Ig negative
909 DPD	78	0	nt	3	2	6	5 Tumor cells probably S-Ig negative
917 NPD	11	0	nt	0	15	5	5 S-Ig profile of coexisting normal lymphocytes, tumor cells probably S-Ig negative
935 DWD	18	0	nt	1	3	7	10 Tumor cells S-Ig negative
936 NPD	37	4	nt	18	13	16	23 Poly clonal pattern
945 DPD (anterior)	6	0	nt	8	0	4	6 Tumor cells S-Ig negative
1070 NPD	nt	3	0	28	38	46	32 Poly clonal pattern
1063 DPD	90			11	81	59	54 Tumor cells probably IgM or IgG (clonal) pattern
1099 NPD (pleural effusion)	nt	0	0	0	82	95	3 Tumor cells IgM
1114 DPD	nt	0	0	<1	100	0	100 Tumor cells IgM
1133 DPD (pleural effusion)	100	0	0	<1	98	<1	99 Tumor cells IgM
1135 DPD (peritoneal effusion)	100	<1	0	100	0	<1	100 Tumor cells IgG
1147 DPD	18 (5)	(10)	22 (8)	<1 (0)	24 (10)	15 (23)	100 (17) Tumor cells probably IgM or IgG (probably) clonal
1148 DPD	<2	0	0	0	0	0	0 Tumor cells S-Ig negative
1152 DPD (pleural effusion)	2	nt	nt	nt	nt	nt	nt Tumor cells probably S-Ig negative
1164 DPD	54	(<1)	(<1)	(25)	(15)	(70)	(25) Poly clonal pattern
Lymphoma in effusions							
839 diffuse	nt	0	nt	0	95	0	95 Tumor cells IgM
878 diffuse	nt	0	nt	98	95	100	17 Tumor cells probably IgG
910 nodular (pleural effusion)	0	0	nt	0	0	0	0 Tumor cells S-Ig negative
934 diffuse	8	0	nt	7	4	16	4 S-Ig profile of coexisting normal lymphocytes, tumor cells S-Ig negative
937 diffuse (pleural effusion)	nt	11	11	56	18	4	21 S-Ig profile of coexisting normal lymphocytes, tumor cells probably S-Ig negative
1077 diffuse	8	0	0	0	0	30	0 Some tumor cells probably surface α-chain
1145 diffuse	17	0	0	3	10	12	4 S-Ig profile of coexisting normal lymphocytes, tumor cells S-Ig negative
1166 diffuse (pleural effusion)	45	(4)	(0)	(0)	(45)	(60)	(<1) Tumor cells probably IgM
Cancer in							
858 non-spec lymphadenitis	nt	50	nt	0	10	11	0 Isoclonal pattern
874 non-spec lymphadenitis	nt	2	nt	0	0	1	1 No S-Ig positive cells
1065 non-spec lymphadenitis	2	0	1	11	15	19	18 Poly clonal pattern

nt Not tested

1) DWD diffuse well-differentiated DPD diffuse poorly-differentiated NPD nodular poorly-differentiated

2) Figures within parentheses indicate percentage of pos. cells after previous tryptic digestion and incubation for 24 h

larger amounts of β - μ (mean 127 range 19-300 ng/5 $\times 10^5$ cells/48 h) comparable to what is produced by LCL and P11A stimulated tonsil cells (62) and the non-neoplastic lymph nodes in this study. No difference was noted between production in S-Ig positive and S-Ig negative LL and HL cases. Initial cell viability and LI did not seem to influence the rate of β - μ production.

Production of lysozyme The production of lysozyme to the growth medium was tested in eight lymphomas. In the six diffuse, poorly-differentiated LL and the two HL (one S-Ig negative, one S-Ig positive) no production was detected. The positive

control line, produced lysozyme (10 μ g/10⁷ cells \times 48 h) while U 766 B1 S 95 K 56⁷ and Daudi were all non-producers, in agreement with previously reported findings (70-79).

Other Characteristics

DNA synthesis in primary tumor cell suspension (Fig. 2) In the group of LL the LI varied between the extremes 0.4 and 14.2% (mean 3.3%). The variation was greatest among the diffuse poorly-differentiated lymphomas (range 0.4-14.2%). The two diffuse, highly-differentiated lymphomas had LIs 11 and 18%. The four nodular poorly

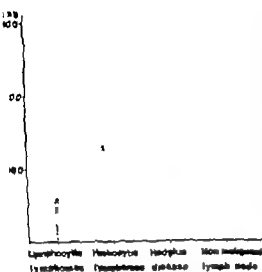


Fig. 1 Production of β -2-microglobulin (ng/5 \times 10⁵ cells \times 48 h) by different lymphomas and control lymph nodes

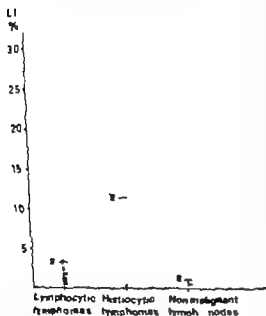


Fig. 2 Labelling index in different lymphomas and control lymph nodes

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Initial cell viability When the tumor-cell suspension had been prepared the cell viability varied greatly (60–100%) between the tumors. The mean initial cell viability for the different types of lymphomas is given in Table 1. No significant differences were observed between the well-differentiated and the poorly-differentiated LL and HL cases. In the cases of HD where a low viability was found, a selective death of large cells was noted.

***In vitro* characteristics** The production of cells from the grids varied considerably among the biopsies, depending on such factors as initial cell viability, amount of tissue explanted, and serum batch in use.

Explanted diffuse LL of both well and poor differentiation generally yielded a homogeneous population of round, mostly non-clumping cells, which attached loosely to the feeder cells. The tumor-cell size varied somewhat with the morphological differentiation. A few non-neoplastic lymphocytes were seen, which often survived longer than the tumor cells. A more heterogeneous cell population was found in nodular LL. The HL generally dislodged large round tumor cells from the grids, which like poorly-differentiated LL did not clump and adhered loosely to feeder cells. In a few cases, however (e.g. biopsy 937) the tumor cells varied somewhat in shape. Round cells were mixed with oval-polygonal cells with short cytoplasmic projections. These histiocytic-like cells did not clump but adhered sometimes to glass and plastic surfaces. The tumor cells in LL and HL survived for a few (2–4) weeks, after which time only non-neoplastic lymphocytes remained in the cultures. The HD biopsies produced a highly heterogeneous cell population from the grids. This population of cells mostly consisted of non-neoplastic lymphocytes of varying shape, often clumping and adhering to the feeder cells. Some large cells were binucleated and resembled Reed-Sternberg cells. They adhered to

differentiated lymphomas varied between 1.2 and 7.9%. Of the five examined HL three exhibited a high LI (31.0, 14.7 and 9.1%), while two cases gave lower values (1.2 and 0.2%). Of the control cells normal peripheral blood lymphocytes gave LIs ranging from 0.1–0.3%. The LIs in patients with infectious mononucleosis ranged from 0.1–3.3%.

TABLE 3 Establishment of Permanent Cell Lines during Long-Term Culture of Malignant Lymphomas

Diagnosis	No. of biopsies effusions	No. of established cell lines (%)	
		LCL	Lymphomas
Lymphocytic lymphomas	22	8 (36%)	7 (5%)
Diffuse well diff	1	0	0
Diffuse poorly diff	17	5	1
Nodular poorly diff	4	3	0
Histiocytic lymphomas	7	1	1
Diffuse	6	1	1
Nodular	1	0	0
Hodgkin's disease	11	10 (91%)	0
Lymphocytic predominance	2	2	
Nodular sclerosis	3	2	
Mixed cellularity	4	4	
Lymphocytic depletion	2	2	
Total	40	19 (48%)	3 (8%)

plastic and glass surfaces, were markedly fragile and did not survive more than few days in the cultures. The production of lymphocytes, however continued for 4-5 weeks, after which time lymphoblastoid cells overgrew other cell types in the grid cultures.

Optimal conditions for the survival in vitro of biopsy cells. The advantage of feeder cells present in the cultures was invariably observed. This prompted us to study the influence of feeder cells, different sera and culture techniques on the proliferation of the tumor cells more systematically. In a series of biopsies the microplate technique described by Epstein and Kaplan (13) was used to study the effect on the survival of tumor cells by different sera (horse, sheep fetal calf, newborn calf, reindeer, rabbit, chicken and human serum, bovine amniotic fluid, lobster hemolymph). None of the sera used was consistently superior to the fetal calf serum. In selected cases different feeder cells (allogeneic fibroblasts or glia cells) in combination with two different calf sera (newborn and fetal) were compared. A variable response to these conditions, again demonstrating the individuality of each tumor with



Fig. 3 The different phases preceding the establishment of the lymphocytic lymphoma line U-698

- a) The cell population produced from grids on day 7. Tumor cells are round and adhere loosely to feeder cells. A few small lymphocytes are present.
 b) After 2 weeks only scarce tumor cells remained.
 c) The established line U-698 after 8 weeks. The line is composed of cells identical to the cells initially produced from the grids.



Fig. 4. Different phases preceding the establishment of the LCL U 704.

a) The cell population produced from the grid carrying the explant (diffuse, poorly-differentiated lymphocytic lymphoma) on day 3. Some tumor cells show pseudopodia on feeder cells, others adhere loosely. Some small lymphocytes are present.

b) A few tumor cells remaining after 3 weeks, loosely adhering to the feeder cells.

c) The established LCL U 704 after 9 weeks with polygonal often pear-shaped cells adhering to feeder cells or seen in clumps.

illustrates the frequency of established cell lines. These lines were of two different types. Three were authentic lymphoma lines while nineteen were of the LCL type. The three lymphoma lines were derived from two diffuse, poorly-differentiated LL (U-698 and U-715) and one diffuse, poorly differentiated HL (U-937). Details concerning the establishment and characteristics of these cell lines have been published (42, 61, 79). Briefly after a period of 2-3 weeks with pronounced death among biopsy cells small, colonies of viable tumor cells were observed loosely attached to the feeder cells. These colonies appeared to grow logarithmically although slow and all three lines could be successfully subcultivated after 40-50 days (Fig. 3). The growth of the lines remained strictly dependent on the presence of feeder cells for several months after the establishment.

The LCL, which represent EBV-carrying, non-neoplastic B-lymphocytes contaminating the tumor explants and effusions, have been thoroughly characterized earlier (63). The establishment of an LCL was signalled by the appearance of pear-shaped, large cells with long pseudopodia as a characteristic morphologic hallmark. These cells were not observed in the cultures during the earliest phases of cultivation. LCL cells were earliest seen as single peripoleptic or free floating cells and in small colonies attaching to the feeder cells (fig. 4). Later peripoleptic cells detached to the medium, where the majority formed typical clumps. The further active growth of these cells was indicated by a drop in medium pH. By this time the cell line could be efficiently subcultivated. The majority of the LCL were derived from HD cases where 10 out of 11 biopsies (91%) gave an LCL. The only HD biopsy which did not give an LCL was a case of nodular sclerosus with dense fibrotic strands and hypocellular tumor tissue.

Eight LCL were established from the 22 cases of LL (36% success rate). These were derived from poorly-differentiated diffuse or nodular lymphomas. Only one LCL was derived from seven HL.

The lag period until an LCL was established varied greatly within the group of HD (mean 58 days, range 22-148 days). For the LL this lag period was equal (mean 57 days), but the variation was less pronounced (range 39-85 days). The one LCL derived from HL was established after 65 days.

DISCUSSION

The conceptual and practical difficulties associated with traditional classifications of malignant non-Hodgkin lymphomas have led to the

regard to nutritional requirements, was noted. In some cases the survival of tumor cells was poor irrespective of the presence of feeder cells and kind of serum. In other cases fetal calf serum and glut cells was superior to other combinations.

Long-term culture. Forty of the 55 tumors were cultivated for periods exceeding 2 months. Table 3

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mean was found in the control group. Significant overlapping, however, was present between the control group and the tumors. Therefore, only a high L1 will indicate malignancy. The figures obtained in this study correspond well with what has been observed in acute leukemias (82, 83) and malignant lymphomas (10). In these reports also a considerable variation between individual cases was observed.

The short-term-culture observations of the lymphoma explants showed that LL and HL yielded a homogeneous tumor-cell population that could be distinguished from the heterogeneous cell population typical of benign lymphoidenopathies. In a few cases the HL explants produced tumor cells that showed similarities to macrophages. This finding seems to confirm the diversified origin of these tumors. Explants from HD and to some extent also nodular LL yielded a heterogeneous cell population in which the tumor-cell population was not easily identified. In these cases no distinguishing features between the lymphomas and reactive conditions were observed except when in the HD cases Reed-Sternberg-resembling cells were identified.

The study of fresh tumor cells from malignant lymphomas is hampered by the difficulty of preparing tumor cell suspensions without contaminating non-neoplastic cells and the rapid death of tumor cells *in vitro*. Obviously by the use of established cell lines such difficulties can be circumvented. However, in contrast to the ease by which tumor lines can be established from Burkitt's lymphomas (2, 3, 14, 21, 39, 43, 53, 63, 69) Non-Burkitt lymphomas only rarely seem to give rise to true tumor lines. Two major obstacles seem to account for this. One is the poor survival of tumor cells *in vitro*. The other one is the overgrowth of the LCL type of cell line. Although 29 attempts were made, only three lymphoma lines were established. This low frequency of success seems to be the general experience.

Although we have no precise explanation for our successful establishment of three lymphoma lines, a few facts may be of significance. The three tumors from which these lines originated were all poorly differentiated. They were radio- and chemotherapy resistant and had a rapidly fatal clinical course due to advanced dissemination. The culture technique used in this study, the gelatin foam (Spongostan) grid culture technique (58), has been successful in establishing LCL (58, 63) and seems at present to be, at least in our experience, the best technique also for establishment of lymphoma lines. A complete medium (Ham's F 10 RPMI 1640) supplemented with calf serum is a prerequisite for the survival and growth of lymphoma cells (61, 79). Also an evident

from data obtained in this study, the presence of feeder cells seems advantageous.

In contrast to the low yield of lymphoma cell lines, LCL were frequently established. This confirms previous reports (11, 56, 58, 60, 63, 68, 81) that LCL readily become established from explants of all types of lymphoma, especially HD.

Although most LL and some HL are of B-lymphocyte origin and therefore may carry receptors for EBV (22, 38, 54) no association with EBV as revealed by the absence of EBNA (72), was found in the tumors investigated in this study. The presence of EBV receptors on tumor cells does not, however, imply that they will be infected when exposed to EBV. This is evidenced by the U-698 cells which have EBV receptors (42) but cannot be superinfected by EBV *in vitro*. Also, the patient from which this cell line originated had a positive anti-EBV titer in serum (Henle, perinatal communication) indicating a previous infection. The exceptional case of Hodgkin's disease (lymphocytic depletion type) in which EBNA positive cells were seen in this study is remarkable. Although serological studies (70, 29, 36, 46, 49, 74) have suggested an association with EBV and this type of HD we regard the finding as no support for an etiological role of EBV in HD since we have no evidence that EBNA was present in tumor-cells and not non-neoplastic cells.

This study was financially supported by the S. Eriksson Cancer Society (project No. 55-873-09XC). The skilful technical assistance by Mrs. Agneta Sahlström and excellent secretarial work by Mrs. Kerstin Lindberg and Miss Ingvald Sol is greatly appreciated. We thank Dr. Dick Kallander, Dr. Lars Kärnäs, Dr. George Klein and Dr. Per Petersson for valuable help.

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several new classifications (48-52) based on a combination of detailed cytological description and characterization by immunological surface markers.

The most frequently used surface marker S-Ig not only indicates B lymphocyte derivation but also gives a hint of the clonality of the tumor. The majority of our diffuse and nodular LL were S-Ig positive, which confirms previous reports (1, 5, 9, 19, 30, 33, 34, 47, 67, 75, 76). One difficulty encountered in our study was the evaluation of the polyclonal S-Ig pattern found in approximately half of the S-Ig positive cases. A polyclonal pattern with presence of two light chains was also observed by a few other authors (5, 76). There are several possible explanations of this fact. (a) Contaminating normal lymphocytes may account for the polyclonal pattern. At least for the solid biopsies from non-Hodgkin's lymphomas this is unlikely since according to histology normal lymphocytes were not present in large numbers. (b) Methodological difficulties may be responsible for the deviations from the expected monoclonal pattern. Antisera may react unspecifically or bind to Fc receptors on the tumor cells. Tests performed to control the mono-specificity of the antisera (double gel diffusion against a panel of human myeloma proteins, staining of cells with known S-Ig profile) gave, however, no indication of non-specific activity. Though the use of F(ab)² fragments would have been preferable to avoid binding of antiserum to Fc receptors, staining of the S-Ig negative and Fc receptor positive cell lines M562 and U 937 was negative with the antisera used. This indicates that binding to Fc receptors was insignificant. (c) Ig molecules may have been passively absorbed to Fc receptors on tumor cells or bound to antigenic determinants *in vivo*. This possibility would have been ruled out with trypsinization of tumor cells prior to S-Ig determination. Unfortunately this procedure could be followed only in very few cases. These cases, however, showed an unaltered staining pattern after the trypsin treatment. (d) Finally, these aneuploid tumors, though of monoclonal origin, may have acquired a polyclonal S-Ig pattern during growth *in vivo* due to their genetic instability. Such drastic changes, however, have not as yet been documented.

In accordance with a few other investigators (1, 5, 47) we found some S-Ig negative cases. Since other surface markers were not tested it could not be determined whether these tumors were derived from B-lymphocytes which did not express S-Ig or if they were of T-lymphocytic, 0-cell or histiocytic origin.

Among the HL half of these cases were S-Ig positive, mostly with a monoclonal pattern. These

were evidently of B-lymphocyte origin, confirming the reports by Cooper *et al* (9), Habeshi and Stuart (23), Morris and Davey (57) and Brown *et al* (6). The remaining half of the histiocytic lymphomas were S-Ig negative. S-Ig negativity of some HL was also described by Aisenberg and Long (11), Cooper *et al* (9) and Gajl Peczkala *et al* (19). The origin of these S-Ig negative cases is uncertain. Though proof is lacking, some of these cases may have represented true histiocytic lymphomas. Further analysis of the histiocytic lymphomas is therefore required, including studies on surface markers, cytochemistry, lysozyme production and phagocytosis. A few small series of HL have been studied utilizing a limited number of these parameters. The results indicate that most histiocytic lymphomas were of either B, T or mixed cell derivation and only a minority were of histiocytic origin (6, 23, 47, 57).

Difficulties in differentiating between malignant and benign conditions on morphological grounds are often encountered in the diagnosis of lymphadenopathies. Other distinguishing parameters are therefore badly needed. Determination of S-Ig pattern and quantitation of cells with receptors for SRBC would be valuable. A monoclonal S-Ig pattern or a homogeneous population of cells with SRBC receptors would definitely favour the diagnosis of a malignant lymphoma. Other tests of possible value to distinguish between malignant and non-neoplastic conditions were examined in this study: β_2 - μ production, incorporation of ³H TdR and the *in vitro* behavior and morphology of explants. β_2 - μ production has previously been found to be low in CLL (17) and non-Hodgkin lymphoma cells (61), whereas a higher production was found in HD (62). Our data, however, revealed no consistent difference in β_2 - μ production between the different categories of lymphomas although a somewhat higher production often was seen among histiocytic lymphomas. Also the β_2 - μ production by lymphocytes from control lymph nodes did not differ from that of the lymphoma cells. It is therefore concluded that the rate of β_2 - μ production is of limited value in discriminating between malignant and non-neoplastic lymph node disorders.

Our data partly confirm the assumption that rate of DNA synthesis is of value in distinguishing malignant and non-neoplastic conditions and as a possible prognostic instrument. Considerable inter-individual variation of the LI was encountered in both groups of lymphomas tested. Highest mean LI was found in histiocytic lymphomas, a fact which seems to reflect the poor prognosis and histologic picture with large pleomorphic tumor cells and frequent mitoses typical of this tumor. The lowest

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INCIDENCE OF SALIVARY GLAND NEOPLASMS IN GREENLAND WITH SPECIAL REFERENCE TO AN ANAPLASTIC CARCINOMA

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Nielsen, N, Højgaard, Mikkelsen, F & Hansen, J ■ Hart Incidence of salivary gland neoplasms in Greenland with special reference to an anaplastic carcinoma. Acta path microbiol scand Sect. A, 86 185-193 1978

Forty-two salivary gland neoplasms were diagnosed in Greenland 1955-1974. Twenty-five cases were malignant of which 92 per cent were an undifferentiated carcinoma, histologically identical to malignant lymphoepithelial lesion. Age adjusted incidence rates for salivary gland carcinomas 1965-1974 were among the highest on record, significantly higher than in Denmark. Prognosis was poor with a 5-year disease-free survival rate of 14 per cent. The majority of previously reported malignant lymphoepithelial lesions of salivary glands have occurred in Arctic dwellers in Alaska and Northern Canada. Virus infection and/or dietary deficiencies may be etiological factors. In the present study secondary nasopharyngeal carcinomas could not always be excluded.

Key words: Salivary gland neoplasms, anaplastic carcinoma, Greenland, eskimos.

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Received 5 x 77 Accepted 1 x 77

In the efforts to elucidate the etiology and pathogenesis of salivary gland neoplasms, information on geographical distribution of this complex group of tumors has proved to be important (37). While a reasonable uniformity in the occurrence of malignant salivary gland neoplasms is found in reports from cancer registries throughout the world (50), the Eskimo populations of Northern Canada and Alaska appear to be an exception. Incidence rates for these population groups have not been published but many reports have substantiated a high relative frequency of malignant salivary gland tumors compared to the total cancer experience (2, 10 19 21 27 29 38,39 47 49).

Since Fibiger (16) in 1923 reported 3 malignant parotid tumors in a series of 14 cancers in Greenland Eskimos, nothing has been published about the occurrence of salivary gland tumors in the Greenland population, the world's largest group of Eskimo origin (40 000 in 1975).

This paper presents incidence and histopathology of salivary gland neoplasms in Greenland in the 20-year period 1955-1974. Some additional clinicopathologic features of malignant cases are reported.

MATERIALS AND METHODS

The Greenland population and the medical facilities in the country have been described in previous publications (23 34).

In the 20-year period under study files from Dronning Ingrid's Hospital and from the district hospitals were reviewed as well as hospital records of Greenland patients admitted to referral centers in Denmark. Pathology reports on surgical biopsy specimens from Greenland were examined, death certificates were reviewed and files from the Danish Cancer Registry were searched for malignant cases reported from Greenland. Clinical findings were analyzed and histologic material reexamined on all patients with salivary gland diseases. In 6 cases, 3 pleomorphic adenomas and 3 anaplastic carcinomas, diagnosed 1955-1959 the original histologic

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TABLE 2 Malignant Salivary Gland Neoplasms in Greenland 1955-1974 Number of Cases by Age and Sex. Age-specific Annual Incidence Rates per 100,000 and Age Adjusted Rates (World and European Standard Populations) Expected Number of Cases 1965-1974 if Rates from Denmark (12) are Applied

Age	1955-1964				1965-1974			
	Cases		Rates		Cases		Rates	
	M	F	M	F	M	F	M	F
10-14	-	-	-	-	2	-	7.6	-
15-19	-	-	-	-	1	-	5.7	-
20-24	-	-	-	-	-	-	-	-
25-29	-	1	-	7.6	-	-	-	-
30-34	-	1	-	8.5	-	4	-	36.4
35-39	1	1	11.6	12.3	1	2	9.5	19.0
40-44	1	1	13.7	14.5	-	-	-	-
45-49	1	-	16.3	-	-	2	-	29.9
50-54	-	-	-	-	1	1	18.7	17.3
55-59	1	-	26.1	-	-	1	-	19.5
60-64	-	-	-	-	-	1	-	24.0
65-69	-	-	-	-	1	-	39.5	-
70	-	-	-	-	-	-	-	-
All ages	4	4	2.4	2.4	6	11	3.1	5.8
Age adjusted rate (w/World)			4.3	3.0			4.5	9.6
Age adjusted rate (w/Europe)			3.5	2.7			3.9	7.7
Expected cases					0.8	1.2		
O/E ratio					7.5	9.2		

The adenoid cystic carcinoma was located in the hard palate of a 54 year old man. Partial resection of the hard and soft palate and partial maxillectomy was followed by irradiation and he is now 6 years later without symptoms.

The remaining 23 malignant cases were undifferentiated, anaplastic carcinomas with specific histologic and clinical features. Microscopically epithelial cells with vesicular nuclei, prominent nuclear membrane and clumped chromatin were arranged in solid cords and nests. No differential structures such as duct, glandular or transitional could be identified nor were epidermoid elements, mucous or remnants of pleomorphic adenomas found. Lymphoid tissue was intimately associated with the epithelial tumor cells in the majority of slides in all cases (Fig. 1). In each of the 23 cases, however, epithelial nests in connective tissue free from lymphoid elements could also be demonstrated. Tumor cells, lymphocytes and plasma cells were often intimately mixed with salivary gland structures (Fig. 2 & Fig. 3).

The histologic picture bore some resemblance to the benign lymphoepithelial lesion described by Gurev (29), and 3 cases were initially misinterpreted as such. One additional case was histologically

misjudged as benign chronic infection. A diagnosis of malignant lymphoepithelioma, primary in salivary glands was made in 2 cases. Metastatic spread to intraportal lymph nodes from unknown primary tumor was considered likely in 5 cases.

Complete physical examination, including ears, nose, and throat, was performed in 17 of the 23 patients with anaplastic carcinoma. In 15 no abnormality was found. Slightly suspicious findings were demonstrated in 2 cases but attempts of biopsy proved successful only after irradiation. Normal microscopic appearance was then present.

Signs of Sjögren's syndrome (rheumatoid arthritis, xerostomia and keratoconjunctivitis sicca) were not identified retrospectively in any patient.

The average delay interval or time between first symptoms and admission to hospital was 15 months for anaplastic carcinomas, varying from 11 days to 4 years. Cervical lymph node metastases were present at admission in 11 cases (48 per cent) and appeared later in 9 (39 per cent). Distant metastases were not demonstrable in any case at admission but appeared later in 10 cases (43 per cent), mainly in lung, liver and bone. However the information at the time of death was often incomplete and only one patient was subjected to

material could no longer be traced but pathology reports contained detailed microscopic descriptions.

Epithelial tumors were grouped according to the World Health Organization classification (46). Incidence rates for malignant salivary gland neoplasms in the two periods 1955-1964 and 1965-1974 were calculated based on the census of 1960 and 1970 respectively. Rates are standardized to «World» and «European» standard populations (13). Age-specific incidence rates for malignant salivary gland tumors in Denmark 1968-1972 (12) were applied to the Greenland population 1965-1974. The expected number of malignant cases thus obtained was compared to the number actually observed (O/E ratio). A rough estimate of the prognosis of malignant anaplastic carcinomas was obtained by the frequency of metastases and by calculation of determinate survival rates during a follow-up period. The determinate survival rate is based on determinate cases, excluding patients lost to follow-up and those dying without signs of tumor disease. For statistical evaluation 95 per cent confidence limit factors for estimates of Poisson distributed variables were used according to *Hoensel et al* (24).

RESULTS

Forty-two salivary gland neoplasms were diagnosed in Greenland in 1955-1974. All cases were histologically confirmed. Forty per cent (17 cases) were benign and 60 per cent (25 cases) malignant (Table 1).

Benign salivary gland neoplasms. Sixteen cases occurred in the native population while one, a pleomorphic adenoma, was located in the submandibular gland of a 31 year-old Danish woman. One mesenchymal tumor, a hemangioma in the parotid gland of a new born native boy, is not included in this group of true salivary gland origin.

Benign tumors occurred 4 times as frequent in the parotid as in the submandibular gland (Table 1). Pleomorphic adenoma predominated with 15 cases

(88 per cent) of which 11 were diagnosed in women. Five pleomorphic adenomas recurred during the period of study. No carcinoma in pleomorphic adenoma was found.

Two cases of papillary cystadenolymphoma bot occurred in males aged 35 and 42. Mean age of the entire group was 46 years, of pleomorphic adenomas alone 47 years. The youngest patient was 1 years old and the oldest 71, both females with pleomorphic adenomas.

Malignant salivary gland neoplasms. Eight malignant tumors were diagnosed in 1955-1964 and 17 in 1965-1974 (Table 2). One anaplastic carcinoma of doubtful salivary gland origin in the palate is not included. The 25 patients, 10 males and 15 females, ranged in age from 12 to 68 years.

Incidence rates 1965-1974, age adjusted to «European» standard population were 4.5 per 100,000 males per annum and 9.6 per 100,000 females; male/female ratio = 1/2. Age-specific incidence rates for females in the same 10-year period peaked in the age group 30-34 with a slight decrease in the older groups (Table 2). O/E ratio 1965-1974 was 7.5 for males and 9.2 for females ($P < 0.05$).

Twenty two malignant cases were located in the parotid gland, 2 in the submandibular gland and 1 in the palate (Table 1). All cases occurred in the native population. Histological classification showed 1 mucoepidermoid tumor, 1 adenoid cystic carcinoma and 23 anaplastic carcinomas.

The mucoepidermoid tumor was diagnosed in the parotid gland of a 12 year old boy. The histologic picture was aggressive with a preponderance of squamous elements. Cellular pleomorphism, prominent nucleoli and mitotic figures were conspicuous and mucicarmine secretory activity was occasionally absent. The patient is presently without evidence of disease 3 years after a total parotidectomy.

TABLE 1 Salivary Gland Neoplasms, Greenland 1955-1974. Distribution of Different Types of Tumors by Location and Sex

	Parotid		Submandibular		Palate		Total	
	M	F	M	F	M	F	M	F
Benign neoplasms	6	7	—	3	—	1	6	11
Pleomorphic adenoma	4	7	—	3	—	1	4	11
Papillary cystadenolymphoma	2	—	—	—	—	—	2	—
Malignant neoplasms	8	14	1	1	1	—	10	15
Anaplastic carcinoma	7	14	1	1	—	—	8	15
Mucoepidermoid carcinoma	1	—	—	—	—	—	1	—
Adenoid cystic carcinoma	—	—	—	—	1	—	1	—

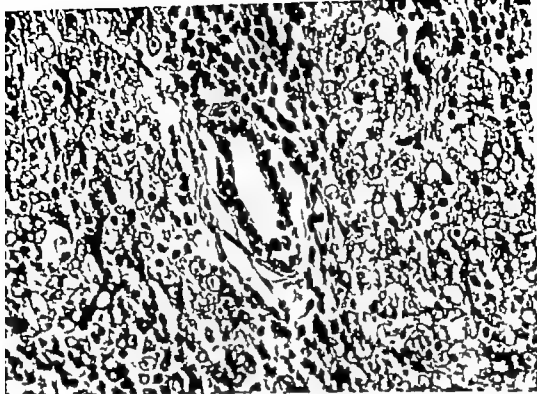


Fig 3 Higher magnification of Fig 2. Note the undifferentiated, pale epithelial cells with large, vesicular nuclei and clumped chromatin. The syncytial nests of tumor cells lie in intimate relationship to lymphocytes. H & E $\times 350$.

post-mortem examination. Of patients with anaplastic carcinoma 3 (13 per cent) received surgical treatment exclusively whereas operation in conjunction with irradiation was performed in 9 (39 per cent) and irradiation alone used in 8 (35 per cent). Three patients received no surgical or radiation treatment.

Three of 22 eligible patients with anaplastic carcinoma were alive 5 years after treatment (Table 3). Five-year determinate survival rate was 14 per cent.

The youngest patient with anaplastic carcinoma was a boy aged 14; the oldest a man aged 68. Two sisters, both midwives, developed anaplastic carcinoma in the left parotid gland at the age of 28 and 34 respectively.

Fig 1 Anaplastic carcinoma of parotid gland initially misinterpreted as benign chronic infection. A few small aggregates of tumor cells disappear in an abundance of lymphoid tissue. H & E $\times 150$.

Fig 2 Lymphocytes and anaplastic tumor cells in total arrangement around parotid gland ducts. H & E $\times 150$.

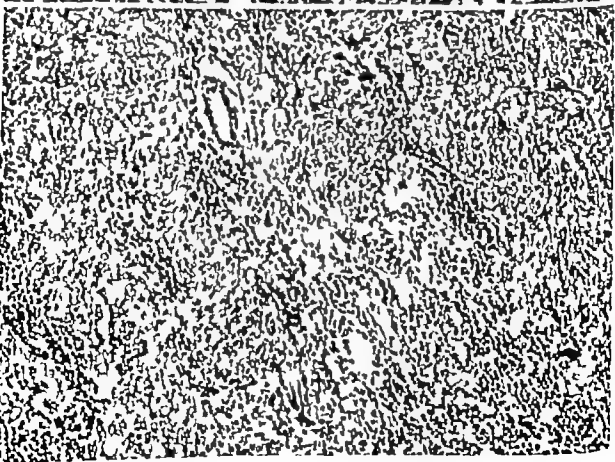
TABLE 3 Anaplastic Carcinoma of Salivary Glands, Greenland 1955-1974. Determinate Survival Rate

Data	Five years	Ten years
Patients eligible	22	14
Patients alive	3	1
Indeterminate	1	0
Determinate survival rate	14 per cent	7 per cent

DISCUSSION

More than 80 per cent of all salivary gland tumors are located in the parotid gland, 5 to 10 per cent are found in the submandibular gland and about 10 per cent in the salivary glands of the palate. Only a few cases occur in the major sublingual gland and in the salivary glands of the tongue, lips, buccal mucosa etc. (14-31).

Most data concerning epithelial tumors of salivary gland origin are available from large hospital series (14-18, 40-45). Approximately 70 per cent prove to be benign, pleomorphic adenomas accoun-



trichomas, histologically similar to those of the recent study has been reported in Canadian and Eskimo *Wallace and co-workers* (49) and 15 salivary gland tumors occurring over a 9 year period in a total Canadian Eskimo population of approximately 11,500. Twelve of these tumors were malignant and 10 were poorly differentiated seroid carcinomas microscopically intimately associated with lymphoid tissue.

Arrheard (2) reported 19 neoplasms occurring in the salivary glands during a 4-year period in the native Alaskan (Eskimo, Indian and Aleut) population estimated at 55,350. Seven patients, 5 Eskimos and 2 Indians, had anaplastic carcinoma of the parotid with a prominent lymphoid background around numerous epithelial islands. All 7 carcinomas were initially confined with the benign lymphoepithelial lesion. Metastatic carcinoma in regional lymph nodes were found in 3 patients at first operation. The author interpreted these tumors as malignant transformations of the epithelial component of benign lymphoepithelial lesions and considered the similar cases previously reported in Canadian Eskimos (49) to have the same pathogenesis.

In a series of 154 parotid gland tumors from Canada *Stula and Buerke* (41) found 3 anaplastic carcinomas, also considered malignant variants of benign lymphoepithelial lesion. The 3 cases were diagnosed in female Canadian Eskimos. Regional metastases were present in two.

The histopathologic category of "benign lymphoepithelial lesions" of salivary gland was created by *Godwin* (20) in 1952 in order to bring together ill-defined conditions previously known as lymphoepithelioma, chronic inflammation, adenolymphoma, lymphocytic tumor and Mikulicz's disease. In spite of diverse clinical presentations and biological course these disorders share a common histologic appearance in the affected salivary gland tissue: a lymphoreticular proliferation associated with atrophy of the acinar parenchyma and ductal changes ending in the so-called myoepithelial island. The unifying benign lymphoepithelial lesion has later been extended to include the salivary gland lesions in Sjögren's syndrome (32).

Well documented extra- and intrasalivary malignant lymphomas have been associated with or have evolved subsequently in cases of histologically substantiated benign lymphoepithelial lesions, with or without evidence of association (1, 5, 26, 28, 36, 44). The first parotid carcinoma considered to be a malignant counterpart of benign lymphoepithelial lesion was described in 1962 (25).

Since then altogether 26 malignant lymphoepithelial lesions have been identified (2, 5, 7, 23, 25,

41, 49), 25 in the parotid and 1 in the submandibular gland. Twenty of the patients, 18 Eskimos and 2 Indians, resided in the North American Arctic of Canada and Alaska. The remaining 6 patients, 2 Caucasians, 2 Negroes and 2 of unmentioned race, were from the U.S. (5, 23, 25) and Norway (7). Manifestations suggesting Sjögren's syndrome were not reported in any of the 26 cases.

The histologic appearance of malignant lymphoepithelial lesion of salivary gland bears a striking resemblance to the malignant lymphoepithelioma of the nasopharynx. Although metastases in general are rare in salivary glands (11, 35, 42), secondary nasopharyngeal cancer in the parotid gland or in preauricular lymph nodes has been reported (17, 22). A high incidence of nasopharyngeal carcinoma has been registered in Greenland (33). In the present study a possible origin from nasopharyngeal cancer can not be totally excluded in all cases even though clinical manifestations of primary lesions outside the salivary glands did not develop during the follow-up. Multiple blind biopsies of the nasopharyngeal region were never taken and irradiation may have obscured the diagnosis.

Little is known about the etiology and pathogenesis of salivary gland tumors. A highly significant increase in salivary gland cancers has been found among survivors of the atomic bombs in Japan (6). Between salivary gland cancer and breast cancer an interesting association has been demonstrated, indicating the possibility of common causal factors (6). No association with breast cancer was found in the present study.

Whether genetic or environmental factors are responsible, the increased incidence in most Eskimo populations and perhaps in all Arctic dwellers (2, 10) is not readily explained. In no other people of Mongoloid stock has a higher than normal rate of malignant salivary gland tumors been reported. The role of Arctic conditions has been evaluated by *Rose and co-workers* (37) who studied the possible co-carcinogenic effect of nutritional, thermal and endocrine factors on chemically induced salivary gland tumors in rats. Increased incidence was found only in vitamin A deficient animals. In a survey of the nutritional status of Alaskan Eskimos (30) clinical signs suggestive of vitamin A deficiency were apparent despite adequate food sources. Similar findings have not been reported in Greenlanders.

The possibility exists that the increased incidence of salivary gland cancer in Eskimos is related only to the anaplastic carcinoma encountered. The histologic resemblance to malignant lymphoepithelioma of the nasopharynx brings to mind this tumor's association with genetic factors and EB-

ting for 50 per cent. Solid undifferentiated anaplastic carcinoma make up from 1 to 22 per cent of all malignant parotid gland neoplasms (4 7 14 41) and account for about 20 per cent in the submandibular gland (14). Anaplastic carcinoma in the palate is usually not considered a salivary gland tumor but placed in the subgroup of epidermoid tumors originating from squamous epithelium of the mucous membrane (14 48).

For anaplastic parotid carcinomas a 5 year determinate survival rate of 32 per cent has been reported (15). On an average 25 per cent of all malignant salivary gland tumors will have spread to surrounding lymph nodes at the time of diagnosis (31). Incidence rates for salivary gland cancers as a compound group are available from 75 population in 28 countries (50). Reported rates seldom exceed 2.0 per 100 000 per annum (Table 4) and pleomorphic adenomas are then often included.

The results of the present study thus differed from previous findings in the following ways.

- 1) A high percentage of all salivary gland tumors registered in Greenland was malignant.
- 2) Age adjusted incidence rates for malignant salivary gland neoplasms in Greenland were high compared with reports from cancer registries throughout the world.
- 3) An unusual distribution of malignant tumor types was found with predominance of anaplastic

carcinomas, exhibiting specific histologic features and a poor prognosis.

As regards the benign tumors of the location and distribution by age and sex follow the normal pattern (40 45). Also the distribution of malignant tumors in the three locations as well as the malignant percentage of cases arising in the submandibular gland (40 per cent) and in the parotid (50 per cent) were consistent with findings in other materials (14 43). In the parotid gland however the number of malignant tumors in relation to all diagnosed parotid gland neoplasms was 63 per cent, 2-3 times higher than reported in most series.

Salivary gland cancers are rarely common tumors which account for less than 1 per cent of all human cancers (31). As in the present study reported incidence rates are often based on test numbers which furthermore include several histologic types (Table 4). Although the age adjusted incidence rates for salivary gland cancers in Greenland are the highest on record (Table 1) caution has to be exercised in the interpretation of such results. However the difference between the observed and the expected number of cases in 1965-1974 is statistically significant. Cancer of salivary glands in this 10-year period was thus significantly commoner in Greenland than in Denmark.

A preponderance of anaplastic salivary gland

TABLE 4 Highest Reported Incidence Rates for Salivary Gland Cancers Age Adjusted to European and World Standard Populations (50) Comparison with Rates from Denmark (12) and Greenland

Population	Period	Number of cases		Rates (European)		Rates (World)	
		M	F	M	F	M	F
Nigeria, Ibadan	1960-69	29	21	2.3	1.7	1.9	1.4
El Paso Spanish population	1968-70	3	3	2.7	1.8	2.0	1.5
Singapore Indians	1968-72	2	-	4.5	-	2.5	-
Saarland, W. Germ.	1968-72	49	50	1.9	1.7	1.5	1.4
New Mexico, white population except Spanish	1969-72	14	19+	1.7	2.0	1.2	1.4
Detroit, Negroes	1969-71	12	6	1.7	0.7	1.3	0.6
Oxford, England	1968-72	87	115+	2.0	2.4	1.5	2.0
Denmark	1968-72	124	145	1.1	1.1	0.8	0.9
Greenland	1965-74	6	11	4.5	9.6	3.9	7.7

+ Pleomorphic adenomas included
More than 10 per cent of unknown age

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virus infection. Certain evidence indicate that virus infection in salivary glands may initiate a progressive sequence of events with reduced secretion rate, retrograde infection and low grade ductal infection as first steps. Further progression can lead to metaplasia of ductal epithelium obstruction chronic recurrent sialadenitis and finally benign lymphoepithelial lesion with or without autoimmunity (4). Malignancy may then arise from either the epithelial or the lymphoreticular components. The same evolutionary spectrum can possibly also be initiated by congenital duct abnormalities (3-9). Studies elucidating the incidence, histopathology and clinical course of non-neoplastic salivary gland diseases in Greenlanders thus appear to be needed.

This work has been supported by Sygekassernes Helsefond, Grant no H 11/51 76

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BRIEF REPORT

LITHIUM INDUCED FOCAL INTERSTITIAL FIBROSIS IN THE RAT KIDNEY

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Hestbeck, J., Olsen, O. V. & Thomsen, K. Lithium-induced focal interstitial fibrosis in the rat kidney. *Acta path. microbiol. scand. Sect. A* 86: 195-197, 1978.

An early stage of focal cortical interstitial fibrosis and nephrotic atrophy was found by light microscopy in the kidneys of nine out of twelve rats which had received lithium for 9 weeks. The animals had excessive polyuria and increased requirement for sodium. The histological changes were in their distribution and the morphological structure similar to the lesions recently found in renal biopsy specimens from patients on long-term lithium treatment.

Key words: Rat kidney, lithium, focal cortical fibrosis, light microscopy.

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Accepted as submitted 18.7.78

It has recently been reported that renal biopsy specimens from 14 patients on long-term lithium treatment with complications such as decreased renal concentrating ability or acute intoxication showed severe focal interstitial cortical fibrosis and nephrotic atrophy (Hestbeck *et al.* 1977).

The aim of the present study was to estimate if prolonged lithium administration to rats might cause similar light-microscopical histologic lesions.

Material and Methods

Male Wistar rats initially weighing 160-170 g. were housed in individual cages in a room with 12-hour light-dark cycle. The animals had free access to a wet mash diet, water and a 7% NaCl solution (Thomsen *et al.* 1974). After one week the rats were divided into six groups of 6 rats each. Groups a, b and c received food containing 0, 80 and 100 $\mu\text{mol/L}$ per kg dry food, respectively, for 1 week. At that time polyuria and inability to retain sodium were fully developed (Thomsen 1971, Jensen *et al.* 1976). Groups A, B, and C received the same treatment for 9 weeks. None of the rats developed severe lithium intoxication characterized by a progressive weight loss and terminal oliguria (Thomsen 1973).

The serum lithium concentrations (mean \pm S.D.) of the groups B and C were 1.1 ± 0.2 $\mu\text{mol/L}$ and 1.3 ± 0.3 $\mu\text{mol/L}$, respectively. The water intake of groups A, B and C (mean \pm S.D.) was 0.34 ± 0.07 , 6.3 ± 0.5 and 7.1 ± 0.9 ml/h/100 g body weight, respectively. The intake of 2.7% NaCl in groups A, B, and C (mean \pm S.D.) was 13 ± 0.9 , 92 ± 10 and 127 ± 22 $\mu\text{mol/h/100 g}$ body weight. The lithium treatment induced hyperexcitability. The weight gain of the lithium rats was about one third of the control rats.

At the end of the treatment the kidneys were removed under amylal anesthesia. Formaldehyde fixed kidney sections were paraffin embedded, cut in 4 μm thick sections and stained with hematoxylin and eosin. Stained for connective tissue and PAS-hematoxylin.

The study was carried out in a blind fashion. Since the kidney size of the lithium rats was smaller than that of the control rats due to lithium-induced growth retardation, the pathologist (J. H.) was not allowed to see the whole kidneys, she was further ignorant of the number of groups of rats.

Results

In 9 of the 12 rats on lithium treatment for nine weeks light-microscopical studies revealed focal lesions scattered in the cortex (Table 1). A lesion (Fig. 1) included from a

es were more pronounced after nine weeks on lithium
administration than after three weeks.
Glomeruli and vessels were normal in all rats.

The main finding of this study was that prolonged
slow administration to rats induced focal cortical
interstitial fibrosis in an early stage. Yavorshki *et al*
(1976) found a considerable sclerosis of the kidneys 30
days after cessation of 6 days of intraperitoneal lithium
administration. The lesions were not focal, and they were
most pronounced in the medulla. A direct comparison
between the findings of Yavorshki *et al* and ours is,
however, not possible due to the different experimental

Yavorshki *et al* administered lithium by hypertonic,
intraperitoneal, toxic doses and at the time of the
examination the rats had recovered from acute renal
failure. In the present study intoxication and acute renal
failure were prevented by giving the rats free access to
sodium chloride (Thomsen *et al* 1974); further lithium
was administered in a continuous way.

The focal cortical lesion of the renal biopsy specimens
from patients on long-term lithium treatment (Hestbeck
et al 1977) is different from the lesion in the rat kidney
as regards the absence of glomerular sclerosis and a
lesser degree of tubular atrophy in the rat. The lesions
are similar in the distribution and the affection of the
tubules, with epithelial changes and thickening of the
basement membranes, and the widening of the intersti-
tium with fibrous tissue in an early stage.

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82: 1780-1782, 1976.

TABLE I *Lithium Induced Structural Alterations*

Group	Total No of rats	Duration of treatment	Lithium dose	Focal cortical lesion	Distal conv tubules	Collecting ducts
		weeks	mmol/kg dry weight of food		Number of rats affected	
a	6	3	0	0	0	1
b	6	3	80	0	6	6
c	6	3	100	0	6	5
A	6	9	0	0	0	1
B	6	9	80	5	6	6
C	6	9	100	4	6	6

few to about 30 tubular profiles: all proximal convoluted tubules, all distal convoluted tubules as well as interstitial tissue were altered. The cytoplasm was basophilic in all the affected tubular profiles. The profiles were smaller than normal with thickened basement membranes but the nuclei were normal. The interstitium in a focal lesion was expanded with edema, mononuclear cell infiltration, fibroblast proliferation and an increased amount of collagen. Focal cortical lesions were found only in rats after nine weeks of lithium administration.

Outside a focal lesion the proximal convoluted tubules and the interstitium were normal while all the distal convoluted tubules in the cortex were distended with epithelial changes. The epithelial cells in the distal convoluted tubules and the collecting ducts were sometimes flat, sometimes swollen without clear delineation of the luminal cell border. There were also variations of size and stainability. The frequency of epithelial lesions appears from Table I. In both the distal convoluted tubules and the collecting ducts the size

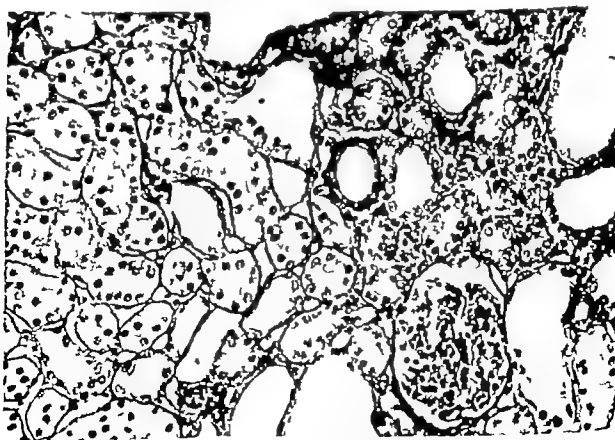


Fig. 1. A focal lesion in the cortex of a rat kidney after nine weeks of lithium administration. The right part shows a section of a focus with small dark proximal tubules in an interstitium with early fibrosis. The left part shows normal proximal tubules. All distal tubules are distended (Sirius red).

lesions were more pronounced after nine weeks on lithium intoxication than after three weeks.

Glomeruli and vessels were normal in all rats.

DISCUSSION

The main finding of this study was that prolonged lithium administration to rats induced focal cortical peritubular fibrosis in an early stage. Yavoraki *et al* (1976) found a considerable sclerosis of the kidneys 30 days after cessation of 6 days of intraperitoneal lithium intoxication. The lesions were not focal, and they were not pronounced in the medulla. A direct comparison between the findings of Yavoraki *et al* and ours is, however, not possible due to the different experimental setups. Yavoraki *et al* administered lithium by hypertonically, intraperitoneal, toxic doses and at the time of the examination the rats had recovered from acute renal failure. In the present study intoxication and acute renal failure were prevented by giving the rats free access to sodium chloride (Thomsen *et al* 1974), further lithium was administered in a continuous way.

The focal cortical lesion of the renal biopsy specimens from patients on long-term lithium treatment (Hestbeck *et al* 1977) is different from the lesion in the rat kidney as regards the absence of glomerular sclerosis and a lesser degree of tubular atrophy in the rat. The lesions are similar in the distribution and the affection of the tubules, with epithelial changes and thickening of the basement membranes, and the widening of the interstitium with fibrous tissue in an early stage.

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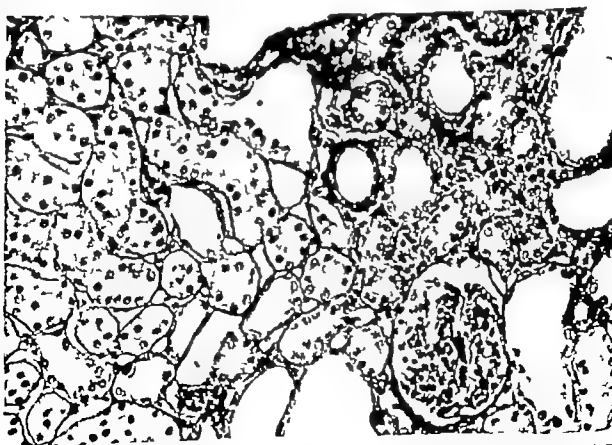


Fig 1 A focal lesion in the cortex of a rat kidney after nine weeks of lithium administration. The right part shows section of a focus with small dark proximal tubuli in an interstitium with early fibrosis. The left part shows normal proximal tubules. All distal tubules are distended (Sirius-red).

BRIEF REPORT

ACUTE HYPERTENSIVE DAMAGE OF ARTERIAL VESSELS OF THE HEART

Finn Olsen

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Olsen, F. Acute hypertensive damage of arterial vessels of the heart. *Acta path microbiol. scand Sect. A*, 86: 199-200 1978

Experimentally-induced acute angiotensin hypertension has been shown to increase the permeability of arterial arteries and arterioles to plasma components within a few hours. Only in one of the coronary arteries was an increased and focal permeability for plasma components demonstrated, the penetration taking place into the entire thickness of the vessel wall. However the permeability observed in intramyocardial small arteries and arterioles showed a distinct deposition of plasma components, both in the tunica intima and the tunica media and usually in the entire circumference of the vessels. The results support the view that an increased arterial permeability of the myocardial vessels for plasma components is an important initial stage in the development of hypertensive vascular disease of the heart.

Key words: Hypertensive damage, acute, arterial vessels, heart.

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Received 2 iv 78 Accepted 2 iv 78

Some of the serious complications associated with arterial hypertension are coronary atherosclerosis, myocardial infarction and congestive heart-failure. The Cause of these complications is chiefly the hypertensive vascular damage, accompanied by a secondary impairment of the function of the heart.

The intention of the present work was to study the initial stages in the development of hypertensive vascular disease of the heart. In particular it was interesting to find out whether it is possible to recognize changes in the permeability of arteries and arterioles in the heart during a few hours of experimentally-induced acute hypertension, as has been demonstrated in the mesentery, the pancreas and the aorta (Giese 1961, 1964; Olsen 1968, 1976; Goldfarb & Berlin 1972; Thorhall & Olsen 1974).

Material and methods

Animals. White female rats, weighing about 150 grams, were used.

Anaesthesia. A solution of amylbarbitone sodium (Læmlyl [®]) 25 mg/ml was injected intraperitoneally at the rate of about $\frac{1}{2}$ ml per 100 grams of rat.

Fluorescent serum proteins. were produced as previously described (Olsen 1968), the procedure was a modification of Neave's method (1962).

Angiotensin. Hypertension CIBA was dissolved in physiological saline.

The experimental technique. Group I. After induction of complete anaesthesia, a catheter of polyethylene was placed in the femoral vein of each of 10 rats, and about 40 mg of Lissamine Rhodamine labelled homologous serum proteins were injected intravenously. About ten minutes later intravenous injections of angiotensin were started in doses of about $\frac{1}{2}$ microgram each, every fifth minute during four hours. By procedure, the blood pressure rose from the normal level (about 90 mm Hg) to 150-160 mm Hg after each injection, and normalized before the next angiotensin injection. After the end of the experimental period the rats were killed by injecting a large dose of amylal intraperitoneally and the heart was fixed in 4% buffered formalin, pH 7.3.

Group II. control animals. Six rats, otherwise treated in the same way as the experimental rats in group I, were given intravenous physiological saline injections instead of angiotensin, the volumes being identical.

Macroscopical preparations. After fixation, the heart was embedded in paraffin, cut into sections five microns thick and examined under a fluorescence microscope. A total of about 300 sections from each rat heart were examined.

Results

Group I. The coronary arteries. Penetration of fluorescent proteins into the walls of the coronary arteries was observed in only one case. This permeability was focal

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Material and methods

Animals. White female rats, weighing about 150 grams, were used.

Anesthesia. A solution of azytuberbutone sodium (Laryol 0.15 mg/ml) was injected intraperitoneally at the rate of about $\frac{1}{2}$ ml per 100 grams of rat.

Fluorescent serum proteins were produced as previously described (Olsen 1964), the procedure was a modification of Mason's method (1962).

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MICRONUCLEI STUDIED IN FINE NEEDLE GOITRE ASPIRATES

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Nilsson, G. Micronuclei studied in fine needle goitre aspirates. *Acta path. microbiol. scand. Sect. A*, 86: 201-204, 1978.

Small nuclei at the size level of 1-3 μ - micronuclei - were studied in fine needle goitre aspirates. The greatest frequency of such structures was found in carcinomas, toxic goitres, and lymphoid thyroiditis, but even in the cases where they were found in greatest numbers, these structures comprised only a few per cent of the nuclei. The possible significance of the micronuclei, especially with regard to carcinogenesis, is discussed.

Key words: Fine needle aspirates, goitre, micronuclei.

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Received 10 77 Accepted 22 77

The nuclei of the follicular epithelium of the thyroid are usually fairly equal in size. One obvious exception is a small number of large nuclei in separate size-classes, found especially in lymphoid goitres and some toxic goitres (7, 8). In the present report the author would like to describe another exception from the main bulk of fairly equally-sized nuclei, namely extremely small nuclei, micronuclei, occasionally found in goitre aspirates (Figs 1-4). These structures are easily studied in fine needle aspirates as opposed to histological sections where small cut-off segments from larger nuclei may give an artifactual appearance of micronuclei.

The author would like to define micronuclei as sharply demarcated structures with the staining characteristics of cell nuclei and with a diameter of about 1-2 μ in separate smears. This size-level contrasts strikingly with that of the nuclei of the ordinary follicular epithelium, which have an average diameter of about 10 μ in smears. The present report concerns an examination of the frequency of micronuclei in various types of goitre aspirates.

MATERIAL AND METHODS

The present report was based on findings of micronuclei in routine clinical diagnostic examination of 600 fine needle goitre aspirates. The material included 78

lymphoid goitres, 132 toxic goitres, 4 cases of sub-acute thyroiditis and 25 carcinomas, which were all of papillary follicular or medullary type. The classification of tumours was confirmed by examination of histological sections from surgical specimens. The rest of the aspirates examined (361 cases) represented a sample goitre cytology with a rather unspecific cytological finding mainly of the type 'colloid goitre' with abundant colloid background substance in the aspirates.

The biopsies were performed as described by Söderström¹⁰ using fine needles with an outer diameter of 0.6-0.7 mm. The smears were dried in air and routinely stained with the May-Grunwald-Giemsa technique. During examination of the smears special attention was paid to the presence of different nuclear size-classes.

RESULTS

Micronuclei (Figs 1-4) were an infrequent finding in all types of goitre studied. They were most often registered in cases of carcinomas (11 of 25 cases), lymphoid goitres (25 of 78 cases), and toxic goitres (22 of 132 cases). In the four cases of sub-acute thyroiditis no micronuclei were found. In other types of goitres they were seldom found (10 of 361 cases). Even in the aspirate smears with the largest frequency of micronuclei these structures comprised only a few per cent of the nuclei. The diameter of



Fig 1 Coronary artery L, lumen. Fluorescent proteins are deposited in the tunica intima and the tunica media (arrow) Magnification 1000 \times



Fig 2 Small intramyocardial artery with a dense deposition of fluorescent proteins in the tunica intima and the tunica media in the entire circumference of the vessel Magnification 1000 \times

and took place in the tunica intima and the tunica media (Fig 1).

Intramyocardial arteries and arterioles. In contrast to the coronary arteries many of the intramyocardial small arteries and arterioles with a diameter from 50 microns to 200 microns showed a marked deposition of fluorescent proteins in the walls. The deposition was localized in the tunica intima and the tunica media and was generally seen throughout the entire outer sheath of the arteries and arterioles (Fig 2). The permeability of arteries with a diameter larger than 200–300 microns was very sparse and was observed in only few cases.

Group II (the control rats)

No deposition of fluorescent proteins was observed in the coronary arteries, the small intramyocardial arteries or the arterioles.

Discussion

The results demonstrate a marked increased permeability for plasma components into the walls of small arteries and arterioles of the heart during acute angiotensin hypertension in rats. By contrast, the permeability of large intramyocardial arteries and the coronary arteries was very sparse and only in a few cases was it possible to ascertain that an increased permeability of fluorescent proteins had taken place into the walls of these vessels.

In this respect, small arteries and arterioles in the heart showed exactly the same increase in permeability for plasma components during acute angiotensin hypertension as had previously been demonstrated for the small arteries and arterioles in the pancreas and the mesentery (Giese 1961, 1964; Olsen 1968; Goldby & Bellin 1972; Thorball & Olsen 1974).

It is a well known observation in cases of sub-acute

and chronic experimental hypertension that widespread arterial lesions are found in the heart (Hägg & Jör 1939; Koletsky 1957; Bein *et al.* 1957), in some cases together with focal myocardial necroses (Selig 1959; Masson *et al.* 1950). The pathogenesis of hypertensive vascular disease of the heart has not been elucidated in these experimental studies, but in cases of chronic rat hypertension in rats it was observed that 125I-fibrin injected intravenously deposited in the arterial walls the myocardium (Kunz *et al.* 1973). The results do support the view that an increased arterial permeability of the myocardial vessels for plasma components is an important initial stage in the development of hypertensive vascular disease of the heart.

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Figs 1-4 Micro nuclei indicated by arrows in May Grönwald-Lacocca stained fine needle aspirates from a muc goitre (Fig. 1), a lymphoid goitre (Fig. 2) with typical Atkinson cell degeneration, and a papillary carcinoma (Fig. 4). Magnification: Fig. 1 and 2 $\times 1600$, Fig. 3 $\times 800$, Fig. 4 $\times 1000$.

the micronuclei were about 1-3 μ , and furthermore here tended to be a sharp upper limit to the size of micronuclei, thereby creating a distance between these nuclei and other nuclei of the follicular epithelium, the diameter of which was about 10 μ . In order to confirm the impression of micronuclei as a special nuclear size-class, the diameters of nuclei not classified as micronuclei were measured with an ocular micrometer. The mean length of the longest diameter and its perpendicular diameter was calculated. This mean diameter was never smaller than 6 μ in 150 nuclei of the follicular epithelium from each of 3 toxic goitres, 3 papillary carcinomas, 3 lymphoid goitres and 3 toxic colloid goitres.

It is noteworthy that the size distance between ordinary cell nuclei and micronuclei could be noted also in the carcinoma aspirates. This may be due to the lack of anaplastic carcinomas in this series. It is conceivable that the marked continuous atrophicavoids in the latter carcinoma type makes it difficult to define a special class of micronuclei.

The neighbour nuclei of the micronuclei, as well as the micronuclei themselves, showed no condensed chromatin structures indicating karyorrhectic. The micronuclei were most often found singly but two micronuclei in the same cell group were occasionally found.

Cell boundaries can only occasionally be delineated in aspirate smears. When cell boundaries could be distinguished (Fig. 3) it was invariably evident that the micronuclei were not part of separate microclones.

DISCUSSION

The origin of micronuclei is difficult to determine at our present stage of knowledge. They may be a product of atrophic separation of nuclear segments from ordinary cell nuclei. Another possible formation mechanism is a disorder of the mitotic phase of the cell cycle. It is thus easy to imagine that the temporary disappearance of the nuclear membrane and the freely-regulated, complicated movement patterns of the chromosomes during mitosis occasionally might result in the separation of one or more chromosomes from the bulk of the chromosomes, thereby giving rise to micronuclei in the intermitotic phase. Micronuclei formed during mitosis of the erythroblast and ultimately retained as Howell-Jolly

bodies in the erythrocytes are thought to be formed in this fashion⁽¹⁾. This special type of micronuclei is of great importance in modern cytogenetic research⁽²⁾.

Independent of whether the micronuclei are formed during the mitotic or the intermitotic phase, they are apt to contribute to a genetic imbalance which may be of importance in the carcinogenetic process. It has been found in many studies that malignancy is associated with aneuploid cell lines^(3,4) and a corresponding variation of the nuclear DNA values has also been demonstrated⁽⁵⁾. The development of such aneuploid cell lines during carcinogenesis is especially well-documented in the thyroid through the studies of iodine-deficient rat goitres^(1, 6). Such aneuploid cell-lines are also found in human goitres during hyperplasia of the follicular epithelium in Hashimoto goitres, toxic goitres, and thyroid carcinomas⁽⁷⁾ i.e. situations where micronuclei are most often found. It is therefore easy to imagine a role of the micronuclei in the formation of aneuploid cell-lines, an implication which evidently gives special interest to these structures in studies of the carcinogenesis of the thyroid.

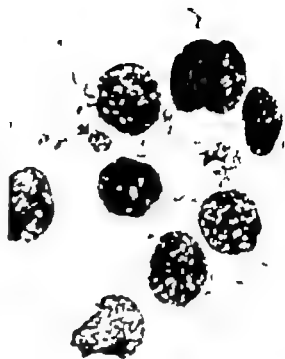
The work was supported by a grant from Axel Linde's Foundation.

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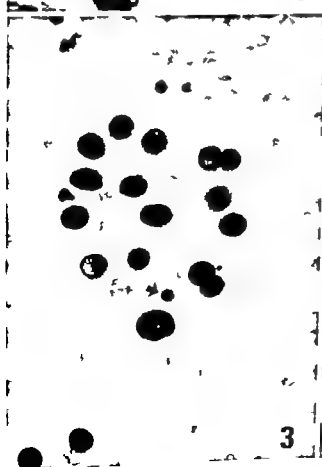
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LYMPH NODE IDENTIFICATION IN CARCINOMA OF THE COLON AND RECTUM

Value of Tissue Specimen Radiography

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Jensen, J. & Andersen, J. Lymph node identification in carcinoma of the colon and rectum. Value of tissue specimen radiography. Acta path. microbiol. scand. Sect. A, 86 205-209 1978.

A method for identifying lymph nodes in tissue specimens from patients with carcinoma of the colon and rectum by soft X-rays is described and assessed. The method seems superior to more time-consuming and possibly tissue-damaging procedures, and it affords a possibility of identifying lymph nodes down to size of 1-2 mm in formalin-fixed as well as in non-fixed tissue specimens.

Key words: Lymph nodes, specimen radiography, carcinoma, colon, rectum.

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Received 24.1.77 Accepted 23.10.77

Demonstrating lymph nodes with metastases in carcinoma of the colon and rectum is of great prognostic importance (7). In studies aiming at an exhaustive description of lymph nodes with a view to ascertaining whether or not metastases are present, the lymph nodes have been isolated by techniques which are time-consuming and, in particular, not applicable for routine investigations (5, 8). Interest in the histomorphological response in lymph nodes draining carcinomas increases the need for a method which is relatively quick and which can locate the lymph nodes in an operation specimen without damaging the tissue.

We have previously (1) presented a method using soft X-rays for locating lymph nodes in specimens predominated by fat. In the present study we used this method for locating lymph nodes in operation specimens from the colon and rectum.

MATERIAL AND METHODS

The material comprises ten specimens of the colon and rectum collected consecutively in our Department. Table 1 presents the material, stating the size of the tumour, the

length of the specimen after 24 hours fixation in formalin, and Duke's stage. The amount of mesentery and/or adventitia was assessed: + signifies a width up to 2 cm, ++ from 2-4 cm, and +++ more than 4 cm.

TABLE 1 Site of Tumour, Size of Gut and Mesentery and/or Adventitia, and Stage of Tumour in 10 Cases of Carcinoma of the Colon and Rectum

No.	Site	Length (cm)	Mesentery and/or adventitia	Stage (1)
419/77	Rectum	36		
852/77	Rectum	22	++	C
1352/77	Asc. colon	28	+	C
1662/77	Rectum	26	++	C
1713/77	Rectum	35	+	B
2109/77	Sigmoid	16	+++	B
2127/77	Caecum	17	++	C
2317/77	Rectum	26	+	C
2721/77	Rectum	15	++	C
2810/77	Sigmoid	14	+	C

(1) Duke's staging

Used as a direct adjunct in clinical diagnostic work
Almqvist & Wiksell Stockholm 1966

- 10 Wu C Al Sadi A A Ling R C & Mc Kinnle
K L Some biochemical and cytogenetic properties
of rat thyroid tumors. Cancer Res. 31 577-587
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F 2 Occurrence of Lymph Nodes, Lymph Nodes with Metastases and Tissue Falsely Interpreted as Lymph Nodes in 10 Cases of Carcinoma of the Colon and Rectum

No	Lymph nodes found by radiography	Lymph nodes found by step sectioning	Lymph nodes found at review	Lymph nodes with metastases	Tissue falsely interpreted as lymph nodes
419/77	54	2	1	7	8
852/77	45	0	0	17	15
1352/77	54	0	2	5	18
1662/77	71	9	0	1	20
1713/77	89	2	0	0	10
2109/77	85	2	0	0	4
2127/77	43	0	1	4	7
2317/77	57	0	1	1	8
2721/77	97	0	0	4	12
2810/77	60	0	0	5	5
Total	655	15	5	44	108

TABLE 3 Size of Lymph Nodes and Tissue Falsely Interpreted as Lymph Nodes in 10 Cases of Carcinoma of the Colon and Rectum

	Size (mm)								Total
	<2	2-4	4-6	6-8	8-10	10-12	12-14	14-16	
Lymph nodes found by radiography	229	260	112	43	6	2	2	1	655
Lymph nodes with metastases	1	15	15	8	2	1	1	1	44
Tissue falsely interpreted as lymph nodes	78	10	4	0	0	0	0	0	108

only one lymph node with metastases was found out of 57 and 71 radiographically located lymph nodes respectively. In the remaining six cases the number of lymph nodes with metastases ranged from 4 to 17. Lymph nodes with metastases were situated in all cases on a level with or proximal to the tumour. In 108 marked sites no lymph node was found, but a vessel - with or without congestion - more rarely lymphoid infiltrates situated free or at vessels, and in one case a vessel ligature. The size of the lymph nodes measured on the X-ray films is shown in Table 3. Let it be emphasized that all the 15 lymph nodes found

accidentally were less than 1 mm in diameter and such lymph nodes could presumably only have been located by means of extremely detailed techniques, such as step and serial sectioning. The five lymph nodes identified at the second review of the specimens were 1-2 mm, and they cannot be located on the X-ray films. We assume that they are superimposed by vessels. The uncertainty of evaluating the X-ray appearances was most in the case of small lymph nodes, but this is of minor importance, as this uncertainty will be solved by the subsequent microscopic examination. A large proportion of the metastases were found in relatively small lymph

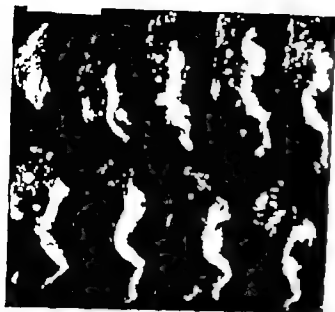


Fig 1 Radiography of 9 slices of a rectal specimen. The slices are oriented identically in the proximal-distal direction from the upper left-hand corner. The lymph nodes manifest themselves as rounded uniformly dense structures on the background of less radiopaque fatty tissue in the mesentery and adventitia. Tumour present in slices 2-9 ($\times 0.5$)



Fig 2 Slice No 9 from Fig 1 exhibiting tumour, lymph nodes and a vessel ($\times 1.2$)

On receipt, the gut was cut open opposite to the mesentery and adventitia and major ligatures of vessels were removed. Thereafter the specimens were cut into slices (mean thickness 7 mm) at right angles to the long axis of the gut. The slices were numbered into the proximal-distal direction and oriented identically on an X-ray film. It was then recorded which slices contained tumour. The X-ray films were exposed in a Faxitron (model 4580SN). This model has a fixed milliamperage of 3, whereas the kilovoltage may be varied in the range 0-110 and the exposure time from 0 to 5 minutes. In the present study we used a kilovoltage between 15 and 25 and an exposure time of 2 min. On the X-ray film (Figs 1 and 2) the lymph nodes may be identified as rounded, smooth, uniform structures on the background of the less radiopaque fatty tissue. The lymph nodes in the individual slices were numbered and their diameter measured on the X-ray film. Thereafter the lymph nodes were isolated by the aid of the X-ray films. It was recorded where a lymph node had been cut and was present in two slices. Whenever a lymph node could be identified macroscopically, only this node was removed for microscopic examination. Lymph nodes measuring less than 6 mm were divided at right angles to their longest axis, and in the case of lymph nodes exceeding 6 mm in size a corresponding central slice was removed for microscopic evaluation (4). Only one histological section was made. When it was not possible to isolate a

lymph node in a marked site macroscopically the entire area was removed for histological evaluation after step sectioning (6-10 levels). Thereafter the remainder of the tissue specimen was again scrutinized by an experienced pathologist (U.A.) who checked, by palpation and transillumination after supplementary incisions through the mesentery and adventitia, whether lymph nodes had been left behind. This review was done without knowledge of the X-ray appearances.

Pretumed lymph nodes found to be completely transformed by metastasis, with perinodal growth, are included in the following analysis as lymph nodes when lying separate from the tumour.

RESULTS

A total of 675 lymph nodes were found. Thereof 655 were located by the aid of radiography, 15 were found accidentally in the step sectioning, and at the second review of the tissue specimens another five could be identified. Table 2 lists the findings in the individual specimens, the number of lymph nodes with metastases, and the number of false positive lymph nodes.

In two specimens it was not possible to find lymph nodes with metastases, and in another two



Fig 5 X-ray film of a slice from a specimen of the colon. A lymph node with metastases and perinodal growth. Partly superimposed (w/o lymph nodes without metastases). The tumour area is also visible (x 1.6)

the area on a level with the tumour as well as the intermediate and principal lymph nodes which drain the tumour area. For research purposes we find the method applicable for approaching in a reasonable manner an exhaustive lymph-node identification without utilizing step or serial sectioning of the entire specimen. Besides, the method causes little damage to the tissue, if it is a description of immunological changes in the lymph nodes which is desired, and it is applicable also to non-fixed operation specimens.

On the other hand, the technique involves certain problems which must be borne in mind. Thus, some of the structures, marked as lymph nodes proved to be other structures, mainly vessels, and this applied especially to nodes which had been thought to represent small lymph nodes. Among larger struc-

tures which may give rise to misinterpretation on the X-ray films there are diverticula which manifest themselves as flask-shaped pouching of the gut (Fig 4). Furthermore it is not possible to determine the number of lymph nodes in cases of superimposition and in the event of lymph-node conglomerates this has to be left to the subsequent dissection and microscopic examination.

At the institution of our study we had planned assessing whether it was possible to point out by radiography lymph nodes with metastases owing to their size, density and possible perinodal growth. This proved possible only to a minor extent. Size and radiopacity proved to be entirely uncertain parameters (cf Table 3), whereas perinodal growth (Fig 5) proved applicable in a small number of cases. In addition, it must be mentioned that one is biased in assessing the tumour area, as it is easily recognized on the X-ray films (Fig. 1).

We must conclude that specimen radiography is well-suited for locating and identifying lymph nodes in carcinoma of the colon and rectum and that the method appears to be superior to more time-consuming and possibly tissue-damaging methods.

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Fig 3 Histological section of lymph node with minimal metastasis. Size 2 mm ($\times 57$).

nodes (Fig 3 Table 3) but it should be noted that the per cent of positive nodes was lower in small lymphnodes.

DISCUSSION

In routine examinations of 10 specimens of the colon and rectum during the preceding year only 29 lymph nodes in all were located and removed and in only three of these specimens they were found to contain metastases. Previous workers have found an extremely varied number of lymph nodes by different techniques. *Dukes* (6) found an average of 17.4 lymph nodes in 596 rectal specimens using very careful dissection, palpation, and transillumination. By the same technique *Gikhrst* and *David* (8) found an average of 23.9 lymph nodes in 22 rectal specimens, but by a modified *Spalteholz* technique, clearing the fatty tissue they could identify an average of 52.1 lymph nodes in 25 rectal specimens. *Collier Kan* and *MacIntyre* (2) who also cleared the fatty tissue, found an average of 67 lymph nodes in 53 rectal specimens and in another study (3) an average of 47.9 in 46 colonic specimens. In this latter study (3) they found a higher average, 52.07 as compared with 41.4 lymph nodes when nodal metastases were present. *Grinnell* (9) studying 322 specimens of the colon and rectum by clearing the fatty tissue, identified an

average of 38 lymph nodes. In our material we found an average of 65.5 by the aid of the X-ray films (range 43-97) in 10 specimens of the colon and rectum.

None of the named authors has mentioned the length of the specimens studied, let alone the amount of mesentery and adventitia. This must of course influence the number of lymph nodes that can be found and therefore comparisons carry some uncertainty. It is our impression that in our material there was on the whole little mesentery and adventitia. Thus, the most peripheral interdigestive lymph nodes were not found to be represented in any specimen. Even though the mesentery and adventitia in the named studies were of the same order of magnitude, the present method represents an alternative to lymph node identification which is not time-consuming and does not damage the tissue. Moreover it renders it possible, by the aid of the X-ray films and the marking of major vessels to divide the lymph nodes into prognostic groups (epicolic, paracolic, intermediate, and main group) (3). The method is applicable in routine investigations which may have to be restricted to studying



Fig 4 X-ray film of diverticula in a slice of the sigmoid colon ($\times 16$) manifesting themselves as flask-shaped pouchings.



Fig 5 X-ray film of a thin frozen specimen of the colon. A lymph node with metastases and perinodal growth. Partly superimposed, two lymph nodes without metastases. The tumour area is also visible (x 1.6).

The area on a level with the tumour as well as the intermediate and principal lymph nodes which drain the tumour area. For research purposes we find the method applicable for approaching, in a reasonable manner, an exhaustive lymph-node identification without utilizing step or serial sectioning of the entire specimen. Besides, the method causes little damage to the tissue, if it is a description of immunological changes in the lymph nodes which is desired and it is applicable also to non-fixed operation specimens.

On the other hand, the technique involves certain problems which must be borne in mind. Thus, some of the structures marked as lymph nodes proved to be other structures, mainly vessels, and this applied especially to ones which had been thought to represent small lymph nodes. Among larger struc-

tures which may give rise to misinterpretation on the X-ray films there are diverticula which manifest themselves as flask-shaped pouching of the gut (Fig. 4). Furthermore, it is not possible to determine the number of lymph nodes in cases of superimposition and in the event of lymph-node conglomerates, this has to be left to the subsequent dissection and microscopic examination.

At the institution of our study we had planned assessing whether it was possible to point out by radiography lymph nodes with metastases owing to their size, density and possible perinodal growth. This proved possible only in a minor extent. Size and radiopacity proved to be entirely uncertain parameters (cf. Table 3), whereas perinodal growth (Fig. 5) proved applicable in a small number of cases. In addition, it must be mentioned that one is biased in assessing the tumour area, as it is easily recognized on the X-ray films (Fig. 1).

We must conclude that specimen radiography is well-suited for locating and identifying lymph nodes in carcinoma of the colon and rectum and that the method appears to be superior to more time-consuming and possibly tissue-damaging methods.

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THE EFFECTS OF ETHYLENE 1-HYDROXY 1-DIPHOSPHONATE ON CELLULAR TRANSFORMATION AND ORGANIC MATRIX OF THE EPIPHYSEAL GROWTH PLATE OF THE RAT - A LIGHT MICROSCOPIC AND ULTRASTRUCTURAL STUDY

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Larsson Å & Larsson S-E. The effects of ethylene-1-hydroxy 1-diphosphonate on cellular transformation and organic matrix of the epiphyseal growth plate of the rat - a light microscopic and ultrastructural study. *Acta path. microbiol. scand. Sect. A*, 86: 211-223 1978

Ethylene-1-hydroxy 1-diphosphonate (EHDP) was administered intraperitoneally to one-day-old rats. In the first experiment, each animal was given 4 injections of EHDP distributed over two consecutive days and corresponding to a total amount of EHDP of 100 mg/kg bwt/day. The animals were sacrificed 12 hours after the last injection. In the second experiment, each animal was given 3 injections, each containing 50 mg of EHDP/kg bwt, with an interval of 11 hours and were thereafter left to survive for 4 days. Animals in the first experiment showed in comparison to normal controls an increased width of the hypertrophic zone, lack of calcified septa within the zone of provisional calcification, persistent atypical chondrocytes in the calcification zone and a large number of matrix vesicles lacking in crystals. The ground substance showed an accumulation of fine precipitates apparently representing undegraded aggregates of proteoglycan macromolecules. Animals in the second experiment formed a new apparently normal epiphyseal growth plate, while the whole epiphyseal cartilage formed under the influence of EHDP remained largely unresorbed within the metaphysis. Besides the previously described inhibitory effects of diphosphonates on the crystallization of matrix vesicles and the growth of hydroxyapatite crystals, EHDP at the doses used was found to have a profound inhibitory effect on the differentiation and migration of the epiphyseal chondrocytes as well as on the degradation of proteoglycan macromolecules. The observed inhibition of vascular invasion appears to be related to inhibition of enzymatic degradation of the ground substance, as evidenced by the observation of extracellular hyaline-like bodies in the erosion zone.

Key words: Diphosphonates, cartilage, calcification, matrix.

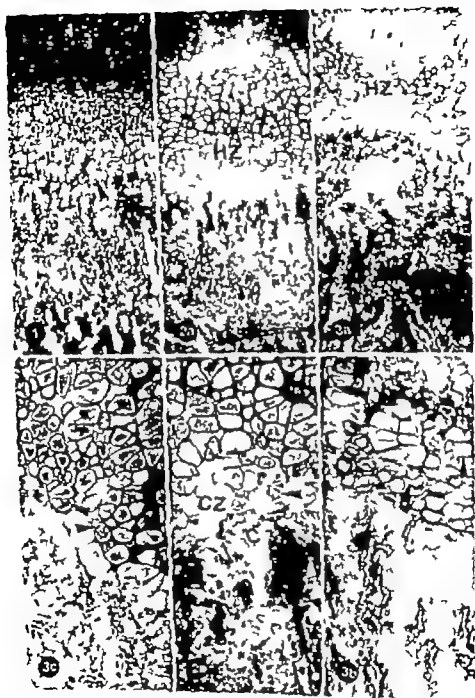
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Received 1 JUL 77 Accepted 1 JUL 77

The synthetic diphosphonates are subject to intensive research because of their relation to pyrophosphates, which have been suggested as possible regulators of bone metabolism (6, 7, 8). The effects of ethylene 1-hydroxy 1-diphosphonate (EHDP) have been explained by an inhibited crystallization of calcium phosphate (8) and a

prevention of crystal growth due to a strong binding on hydroxyapatite crystals (13).

Much of the *in vivo* experimentation with EHDP has been focused around the epiphyseal growth plate, which is a rather complex tissue (e.g., 2, 4). Light microscopic and ultrastructural studies have shown that the effects of EHDP are not merely restricted to an inhibited mineralization and resorp-



tion of bone cartilage and dentin (17, 21, 24) but in calcifying cartilage they are also associated with an inhibition of proteoglycan degradation (18), absence of vascular invasion (24) and persistence of atypical chondrocytes in the calcification zone (18). Inhibited synthesis of glycosaminoglycans (proteoglycans) by EHDP has been demonstrated in rabbit epiphyseal growth cartilage *in vivo* (15). The purpose of the present study was to investigate further the morphologic changes of the epiphyseal growth plate induced by high doses of EHDP, especially with regard to the effects on cell transformation, the degradation of organic matrix and the vascular invasion.

MATERIAL AND METHODS

Two experimental groups of Sprague Dawley rats were used in the present study. In the first experiment, each of 3 one-day-old rats was given 4 intraperitoneal injections of ethylene-1-hydroxy-1,1-diphosphonate (EHDP, a gift from Henkel Cie GmbH, Dusseldorf, West Germany) every morning and evening during 2 consecutive days. EHDP was dissolved in 0.9% NaCl at a concentration of 5 mg/cc and a volume of 0.01 cc/g body weight was administered each time corresponding to an amount of EHDP of 50 mg/kg body weight/injection. Three intact animals served as controls. Twelve hours after the last injection, all the animals were anesthetized with sodium pentobarbital (Nembutal), and fixed by perfusion followed by the processing of the proximal tibial epiphyseal growth plates for electron microscopy as previously described (18).

In the second experiment, each of 3 rats was given 3 intraperitoneal injections of the EHDP solution with a 6-hour interval, while control animals received no injection. The animals were left to survive for 4 days and thereafter the proximal tibial epiphyses were processed for electron microscopy as indicated above. Thick and thin sectioning, staining, contrasting and electron microscopy were carried out as described previously (18).

RESULTS

The light microscopic and ultrastructural architecture of the normal epiphyseal growth plate in the rat has been previously described (18). To facilitate recognition of the disturbances caused by the administration of EHDP in the present study, a light micrograph of a growth plate from a control animal is shown in Fig. 1.

A. Light Microscopic Observations

In rats receiving 4 x 50 mg EHDP/kg body weight and killed the day after the last injection, the width of the hypertrophic zone of the growth cartilage was markedly increased (Fig. 2a). No mineralization of the cartilage septa could be seen

Figs. 1-3 are light micrographs, depicting proximal tibiae of control and EHDP-treated rats.

Fig. 1 Control section, showing the various zones of epiphyseal growth plate and the vascular septa. Epiphyseal-metaphyseal junction is at arrow x100.

Fig. 2a and b From animal receiving 4 x 50 mg EHDP/kg. The hypertrophic zone (HZ) shows an increased width. There is a lack of demonstrable mineralization in the presumptive calcification zone (Fig. 2b CZ). Various cellular elements are present within the lacunae of the epiphyseal-metaphyseal junction (arrow), and arteries (C) have halted in the unmineralized cartilage. 2a x 100, Fig. 2b x 200.

Fig. 3a, b and c From animal receiving 150 mg EHDP/kg and left to survive for five days. A box of unmineralized cartilage appears in the metaphysis (Fig. 3a, aA) separated from the growth cartilage by cellular and vascular zone. Note vascular canal in center of cartilage bar. In the cartilage labelled aA, many numerous lacunae contain cellular elements, some in pairs (Fig. 3b, arrow a). A reduced size of lacunae is seen in the hypertrophic zone (Fig. 3a, HZ), which is widened. Cellular elements are seen in many of the lacunae adjacent to the metaphysis, and some deeply-stained granules are also seen here (Fig. 3b, arrows). There is an almost total lack of mineralization in the presumptive calcification zone as seen in Fig. 3c. Fig. 3a x 100, Fig. 3b and c x 200.

Figs. 4-27 are electron micrographs.

Figs. 4-6 are from animal receiving 4 x 50 mg EHDP/kg and Figs. 7-8 are from control animal.

Fig. 4 Chondrocyte at the epiphyseal-metaphyseal junction. Note well-preserved nucleus and organelles, also the many cytoplasmic processes. x14,800.

Fig. 5 Chondrocyte at the epiphyseal-metaphyseal junction. In contrast to the cell depicted in Fig. 4, this cell is shrunken and numerous granules appear in pericellular lacunae. x7,500.

Fig. 6 Higher magnification of cytoplasmic processes corresponding to that seen in Fig. 5. Note accumulation of granules that lack a limiting membrane and exhibit an amorphous matrix. x50,500.

Fig. 7 Higher magnification of outer surface of chondrocyte in the upper hypertrophic zone of control animal. Small and large granules are present in pericellular space. The small granules are often arranged in rows. The large granules are similar to those depicted in Fig. 6. Both types of granules lack a limiting membrane and exhibit an amorphous matrix. x50,500.

Fig. 8 Hypertrophic chondrocyte of control animal. The cell shows signs of disintegration. Note lack of granules in pericellular space, of EHDP-treated animal. Fig. 5, 6 x 2,700.

(Fig. 2b). The size of the chondrocyte lacunae was reduced, and at the epiphyseal-metaphyseal junction all remnants or even whole cells were frequently observed within lacunae. Toluidine-blue stained granules were a frequent finding within these lacunae. A few slender calcified trabeculae appeared in the metaphysis, and a rim of unmineralized osteoid was seen at the surfaces. The vessels did not penetrate the unmineralized growth cartilage but were strictly localized to the metaphysis (Fig. 2b). In the rats receiving a total amount of 150 mg EHDP/kg body weight and then left to recover for 4 days, extensive changes were seen in the proximal tibia (Fig. 3a). A zone of undegraded, unmineralized cartilage appeared far below in the metaphysis, separated from the recovered, newly formed growth cartilage by a tissue that was rich in mononuclear osteoblast-like cells, widened vascular spaces and badly developed bony trabeculae. Within this persistent cartilage were numerous cell elements were present in the lacunae, with pairs of cells having a similar appearance to that of the chondrocytes in hyaline cartilage (Fig. 3b). The central portion of the cartilage was penetrated by capillaries surrounded by spicules of unmineralized osteoid (Fig. 3a). On the metaphyseal side of the cartilage bar rows of osteoblasts were covering spicules of unmineralized osteoid.

The newly-formed growth cartilage exhibited an increased width of the hypertrophic zone (Fig. 3a). The size of chondrocyte lacunae was reduced, and cell elements were frequently observed in the lacunae closest to the metaphysis. A number of toluidine-blue stained granules appeared within many of these lacunae (Fig. 3c). There was no evidence of capillary invasion at the epiphyseal-metaphyseal junction. Red blood cells were seemingly floating freely in exposed lacunae at the junction. Some mineralization of septa was evident in the areas closest to the periosteal tissues.

B. Electron Microscopic Observations

1/Rats given 4×50 mg EHDP/kg bw: Many of the chondrocytes at the epiphyseal-metaphyseal junction seemed extremely well preserved, with a well-developed rough-surfaced ER, cytoplasmic vesicles and a Golgi complex (Fig. 4). These cells showed an irregular surface, with many short cytoplasmic processes projecting into the pericellular lacuna where a moderate number of small granules could be seen. Other chondrocytes had a shrunken appearance, with irregular nuclei and large cytoplasmic vesicles (Fig. 5). A prominent finding in association with many of the described cells at the epiphyseal-metaphyseal junction was the presence within the pericellular lacunae of nume-

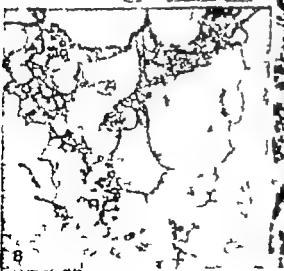
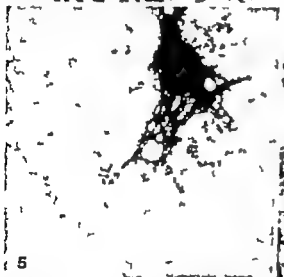
rous granules of a somewhat varying size (Fig. 5). The granules had an amorphous appearance, and no distinct enveloping membrane could be discerned around them (Fig. 6). These granules had an appearance similar to the granules normally found in lacunae at the lower proliferative-upper hypertrophic zone of controls and representing proteoglycan complexes (19) (Fig. 7). Among the latter granules of the controls were also smaller granules, which were often arranged in rows or strings perpendicular to the chondrocyte surfaces (Fig. 7). Such granules were, however, only seen in that zone and not in the lacunae at the lower hypertrophic and calcifying zone of controls, where chondrocytes exhibited signs of extensive disintegration (Fig. 8).

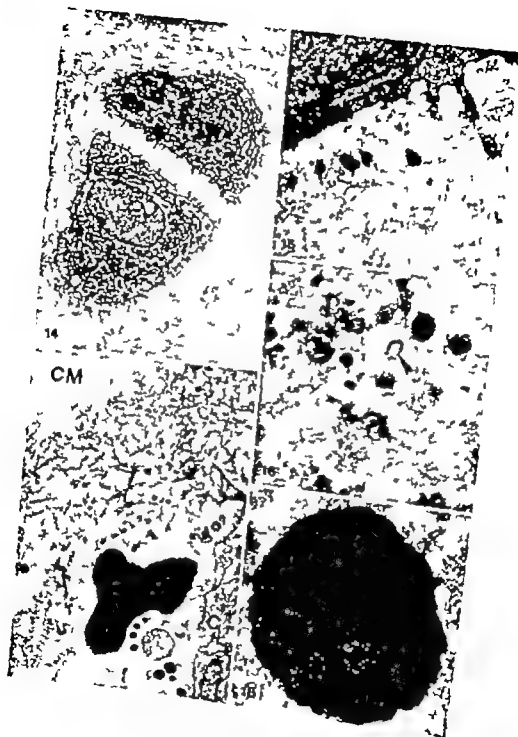
At the light microscopic level, toluidine-blue stained particles were observed in the zones of both controls and EHDP-treated animals, which corresponded to those where the granules appeared at the ultrastructural level.

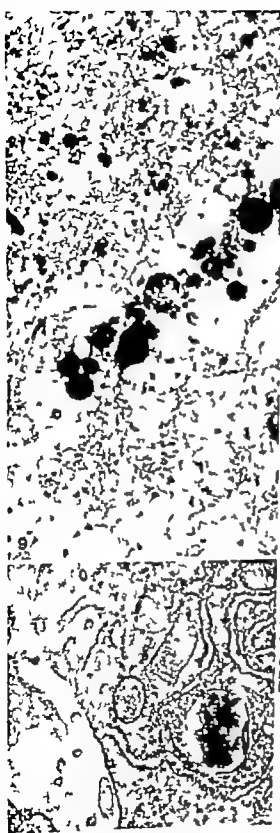
Thick layers of unmineralized cartilage matrix surrounded the lacunae at the epiphyseal metaphyseal junction of the EHDP-treated animals. No clusters of apatite could be seen in this matrix, where mineralization normally takes place in the controls. Matrix vesicles were abundant, however, and they were of varying appearance, mainly corresponding to the morphologic types I and II, as defined by *Thiberg and Friberg* (1972). No crystallites could be observed within any of these vesicles (Fig. 9).

Capillary sprouts were found in intimate contact with the unmineralized cartilage matrix at the epiphyseal-metaphyseal junction of the 4×50 mg EHDP-treated animals. Mononuclear cells were present in many of the vascular lumina. Sometimes these cells were in contact with cartilage matrix at gaps of the endothelial wall. At other places, mononuclear cells appeared outside the vessel wall (Fig. 10) where the cartilage matrix exhibited some interesting features. Small clusters of apatite crystals appeared at irregular intervals in this matrix (Figs. 10-11). Matrix vesicles were also present in the area but no crystallites could be seen within them (Fig. 12). The mononuclear cells exhibited plasma membrane irregularities, and apatite crystals were seen at surface invaginations (Fig. 11). Occasionally crystal clusters were also seen in the cell cytoplasm, bounded by a membrane (Fig. 13).

2/Rats given 3×50 mg EHDP/kg bw: Interest was focused on two areas of the epiphyseal-metaphyseal complex, namely on the persistent cartilage bars observed light microscopically to contain nucleated cells (cf. Fig. 3a) and on the epiphyseal metaphyseal junction (cf. Fig. 3c). In the







Figs 9-13 are from animal receiving 4×50 mg EHDP/kg

Fig 9 From cartilage septa in the zone corresponding to that where calcification normally takes place. A number of matrix vesicles are present in the uncalcified matrix, which also contains a number of fine fibrils and granules. No crystallites can be seen in the vesicles. $\times 26\,700$

Fig 10 Part of vessel wall from tip of capillary vessel opposite unmineralized cartilage matrix. Mononuclear cells (N) appear outside endothelial lining (E). $\times 3\,900$

Fig 11 Higher magnification of outer surface of mononuclear cell depicted in Fig 10. Small clusters of apatite are present in the cartilage matrix and also at cell surface invaginations (arrowheads). $\times 20\,800$

Fig 12 Matrix vesicles outside mononuclear cell depicted in Fig 10. No crystallites can be seen in the vesicles. $\times 22\,000$

Fig 13 Part of cytoplasm of mononuclear cell depicted in Fig 10. An apatite cluster appears within the cytoplasm delimited by a membrane. $\times 22\,000$

Figs. 14-18 are from animal receiving 3×50 mg EHDP/kg

Fig. 14 From cartilage bar depicted in Fig. 3a. Two closely associated cells are embedded in a fine fibrillar matrix and they exhibit a well-developed ER and numerous cytoplasmic projections. $\times 3,200$

Fig. 15 From surface of one of the cells depicted in Fig. 14. Fine fibrils, small granules and cross-sectioned cytoplasmic processes are seen in the pericellular space. $\times 37,000$

Fig. 16 Matrix vesicles appearing in the cartilage matrix surrounding the pericellular space depicted in Figs. 14 and 15. Some of the vesicles have an electron-dense matrix, whereas others look empty (arrow). No crystallites can be seen. $\times 37,000$

Fig. 17 From epiphyseal-metaphyseal junction. A number of extracellular vesicles, erythrocytes and fine fibrils are present in the space between the capillary wall (C) and the uncalcified cartilage matrix (CN). $\times 7,700$

Fig. 18 Higher magnification of vesicle appearing in the extracellular space depicted in Fig. 17. The vesicle is membrane-delimited, and a number of small globular bodies are seen in the amorphous matrix of the vesicle. $\times 5,000$

Fig. 19— are from animal receiving 3×50 mg EHDP/kg

Fig. 19 Higher magnification of vesicle appearing in the extracellular space depicted in Fig. 17. Large, dense granules are present in the matrix of the vesicle. $\times 14,000$

Fig. 20 Chondrocytic lacunae at the epiphyseal-metaphyseal junction. The lacunae is filled with a meshwork of fine fibrils. Granules of an electron-dense material appear in the lacunae and also in the uncalcified cartilage matrix (arrow). $\times 3,900$

Fig. 21 Higher magnification of irregular granules depicted in Fig. 20. The granules lack a distinct limiting membrane. Their matrix is finely granular and a number of fine fibrils and protofilament granules are closely associated with their outer surface. $\times 6,700$

Fig. 22 Matrix vesicles at the epiphyseal-metaphyseal junction. Needle-like crystallites are seen in the vesicles and they are often projecting through the vesicle membrane (arrow). $\times 29,000$

fibrils and small granules could be seen (Fig. 15). In the cartilage matrix, surrounding the lacunar spaces, various types of vesicles were present. Some of these had a homogeneous, electron-dense matrix, whereas others appeared more electron-lucent, sometimes almost to the degree of lacking visible contents (Fig. 16). No crystals could be seen in any of these vesicles.

At the epiphyseal-metaphyseal junction, many of the lacunae contained a mixture of extravasated erythrocytes, membrane-delimited vesicles, bundles of fine filaments and aggregates of a finely stippled material (Fig. 17). The vesicles were of varying appearance and size. The matrix of some of them had a low electron density whereas other vesicles had an electron-dense, finely granular matrix, composed of globular bodies with a diameter of $75\text{--}80\text{\AA}$ (Fig. 18). Occasionally larger granules measuring $300\text{--}400\text{\AA}$ were also present in the matrix of these vesicles (Fig. 19). Most of the vesicles were less than a micron in diameter and mostly they had the more electron-dense type of matrix, with fine granules.

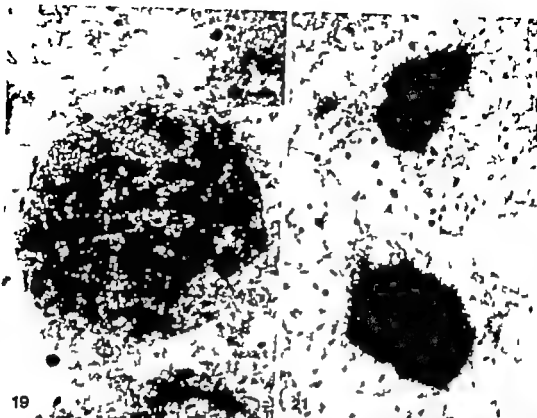
Dense granules were found in many of the chondrocytic lacunae of a type similar to those seen in the corresponding zone of the 4×50 mg EHDP group (cf. Fig. 6). Numerous lacunae in the area were completely filled by a meshwork of fine fibrils and such granules (Fig. 20). The cells in these lacunae were often well preserved, with numerous small cytoplasmic projections. An unusual finding was the appearance of numerous, irregularly formed, large electron-dense bodies, which were not clearly membrane-delimited (Figs. 20, 21). The matrix of these bodies was finely granular and the granules had a size of about 75\AA (Fig. 21). The bodies were present both in the unmineralized cartilage matrix and in the pericellular spaces of the lacunae.

Matrix vesicles were also identified at the epiphyseal-metaphyseal junction of the 3×50 mg EHDP-group. The vesicles were similar to those seen in the corresponding zone of the 4×50 mg EHDP-group and the controls. However, in contrast to those of the 4×50 mg EHDP-group, the matrix vesicles of the 3×50 mg EHDP-group contained needle-like crystallites, often projecting through the vesicle membrane (Fig. 22).

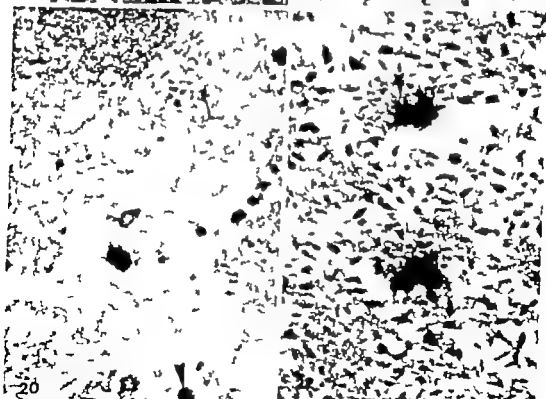
DISCUSSION

The doses of EHDP given intraperitoneally in the present study were much higher than those being currently tested clinically in Paget's disease and other bone disorders (see 22 for a review). From an experimental point of view, the use of high doses of

former area, the cells often appeared separated from each other only by a thin rim of unmineralized cartilage (Fig. 14). Mitochondria, rough-surfaced ER and a discrete Golgi complex were seen in the cytoplasm of these cells. The surface of the cells was irregular with many small cytoplasmic projections extending only a short distance into the pericellular space. In the latter fine



19



20

rabbit growth plate *in vivo* (15). The fact that numerous granules appeared not only in the matrix but also in the pericellular lacunae of the EHDP-treated rats suggests that a continuous but slow synthesis of proteoglycan granules may still take place in these cells or that the secretion and transportation of proteoglycans out into the matrix is blocked. These findings thus indicate a failure of degradation of the proteoglycans as a result of the EHDP-administration. Howell and Pita (1977) reported on the appearance of proteoglycan super aggregates in phosphonate (EHDP) rickets of rat cartilage and presented evidence indicating that such superaggregates may also have a regulatory role *in vivo* by inhibiting mineral phase growth. The large granules seen in the lacunae at the epiphyseal-metaphyseal junction of the EHDP-treated rats are similar to the granules observed in the control animals in the proliferative-upper hypertrophic zone, where proteoglycan synthesis is most active (19). Normally the size of these large granules is rapidly reduced as the granules become incorporated into the matrix (27). In the EHDP-treated rats we also found large aggregates of a granular material never observed in normal animals. We suggest that these granular structures observed at the epiphyseal-metaphyseal junction of the EHDP-treated rats may represent the morphologic counterpart of Howell and Pita's (1977) biochemically-demonstrated superaggregates of proteoglycans formed *in vivo* under the influence of EHDP. The inhibition of crystal formation and growth caused by EHDP may be the result of a primary EHDP-mediated inhibition of proteoglycan degradation (cf. 15).

At the light microscopic level and compared with the controls, the width of the hypertrophic zone was markedly increased in the two experimental groups of the present study; the size of the chondrocytes was reduced, and cells or cell remnants appeared in lacunae at the epiphyseal-metaphyseal junction. These findings are in agreement with previous observations (3, 16, 18, 24). Furthermore, well-preserved atypical chondrocytes were found in unresorbed cartilage bars in the 3 x 50 mg EHDP group, with no evidence of mineralization of the matrix. From the observed effects induced by EHDP it seems that the initial steps of hypertrophy are not detrimentally affected by EHDP, which is also supported by the findings of Bhaz et al. (1975), and that some of the EHDP-induced changes may involve not only an inhibited degradation of the proteoglycans but of the chondrocytes as well.

The mechanism of hypertrophy and disintegration of chondrocytes as an integral part of cartilage calcification is a subject of dispute. Some authors believe that the chondrocyte itself provides the

enzymes necessary for the disintegration of the cells (12). Holtrop (1972), on the other hand, has suggested that the majority of the chondrocytes do not disintegrate but stay intact and transform into bone cells at the calcification and erosion zone. To some extent, this assumption may be supported by some of the present findings. The cells appearing in the undegraded cartilage bars of the 3 x 50 mg EHDP-groups and a number of the cells persisting at the epiphyseal-metaphyseal junction of the animals in both experimental groups, exhibited features of osteoblasts with a well-developed rough-surfaced ER which is not a prominent feature of the hypertrophic chondrocyte (9). Thus it cannot be fully concluded that the cellular EHDP-effects observed in the present and previous studies are solely related to a suppressed disintegration of hypertrophic chondrocytes.

Another interesting point emerging from the present study is the absence of capillary penetration into the unmineralized cartilage. It has been stated (3) that a preceding mineralization is not necessary for the capillary invasion into the epiphyseal growth plate. From our results and others (24), it can be concluded that the penetration of capillaries into epiphyseal cartilage is inhibited by EHDP at doses causing lack of mineralization. The mechanisms underlying the vascular invasion are not known. The vessels are normally accompanied by mononuclear cells which may exert resorptive activity (25). In the present study mononuclear cells were present at the tip of the capillaries in the EHDP-treated rats, and evidence of resorptive activity was also observed. It is not known whether this resorption is an integral part of the vascular invasion or whether the mononuclear cells merely have a scavenging function, engulfing remnants of disintegrated cells and matrix including mineral. Apparently the process of capillary invasion is intimately associated with the activities and fate of the hypertrophic chondrocytes in the calcification zone. The inhibitory effect by EHDP on capillary invasion may therefore be due to inhibition of the activity of the vessel-mononuclear cell complex and/or the described changes of the cartilage matrix-chondrocyte complex.

We consider now that the inhibition of capillary invasion caused by the administration of EHDP is due to a lack of degradation of cartilage matrix components as evidenced by the observed persistence of proteoglycan granules in the matrix and the chondrocyte lacunae. These findings certainly reflect an enzyme inhibitory effect by EHDP. In fact, Howell and Pita (1977) have reported on a total suppression of lyszyme activity which was promptly restored by removal of phosphonate

EHDP may be justified in order to cause distinct morphologic changes which may reflect specific interactions between the compound and the basic processes of the tissue. This information is needed as a basis for further evaluation of the clinical effects of diphosphonates, although any direct extrapolation should be done with care. On the basis of the known physical properties of EHDP studies of such interferences may also contribute to an increased understanding not only of the biological effects of the compound but also of the mechanisms responsible for the function normally exerted by the tissue.

In the present study extensive changes were observed in the epiphyseal growth plate of the two experimental groups. A lack of mineralization of cartilage septa was a prominent finding and toluidine-blue stained undegraded granules were consistently seen in the pericellular lacunae of persistent atypical chondrocytes located at the epiphyseal metaphyseal junction. Similar granules were seen in the controls only in the proliferative and upper hypertrophic zones and not in the narrow zone of calcification. Vascular invasion was inhibited in both experimental groups. In addition to these more apparent changes there was also structural evidence of other serious disturbances by EHDP involving not only the calcification process but also the cellular and vascular activities of the epiphyseal growth plate which altogether indicate that the effects caused by EHDP are complex.

It is known that EHDP given to experimental animals at doses of 10-60 mg/kg bwt/day will result in the formation of a wide osteoid seams and a lack of cartilage mineralization in the epiphyseal growth plate (3, 14, 23, 24) and also in the mandibular condyle (16). Matrix vesicles apparently play an integral part of the mineralization process of epiphyseal growth cartilage, somehow mediating the initial formation of apatite crystals (1). In the present study the uncalcified cartilage septa of EHDP treated animals displayed a large number of such matrix vesicles, which were of normal appearance. Thus, the inhibition of mineralization caused by EHDP cannot be directly related to an absence of a disturbed production of matrix vesicles but merely to an inhibited crystal formation within the vesicles. In the 4 x 50 mg EHDP group maintained on EHDP continuously the vesicles contained no crystals whereas an evident re-appearance of crystals was observed in matrix vesicles of the 3 x 50 mg EHDP-group after a recovery period of four days after the last EHDP injection. These findings seem to indicate that the local concentration of EHDP in epiphyseal cartilage required for the inhibition of crystal formation in

the matrix vesicles is only maintained for relatively short periods of time after the administration of EHDP to the animal. This finding seems to be somewhat at variance with the observation that the bone retention of EHDP is about 2 to 4 weeks (14, 20). However, this calculated figure does not seem to represent the situation at the actual site of calcification, i.e. the osteoid, where the local concentration of EHDP is probably more related to that of the extracellular fluid than to that accumulated in the bone. Furthermore, the appearance of small clusters of apatite in the cartilage matrix, close to the capillaries, in both of our experimental groups indicates that depending upon the diffusion of EHDP in the tissues local very limited tissue sites may still exhibit formation of apatite crystals, certainly due to local variations in EHDP concentration. The conclusion we wish to make is that EHDP inhibits the initial crystal formation within the matrix vesicles and that a resumed mineralization takes place relatively shortly after withdrawal of EHDP. However, the spreading growth of the initially formed crystals is still inhibited by EHDP for an unknown period of time, certainly dependent upon the actual local concentration of EHDP in the tissue.

An accumulation of fine granules was observed by Larsson & Larsson (1976) in the ground substance of epiphyseal cartilage septa of EHDP treated animals, and the appearance and distribution of granules have been further examined in the present study. In EHDP-treated animals, granules remained undegraded in the matrix and in the lacunae of the zone where calcification takes place in the normal controls. Normally the calcification zone does not contain such granules in the chondrocyte lacunae, and their number is also strongly reduced in the cartilage matrix (see also 5, 27). Granules appear normally in our controls as well, in large numbers in the pericellular lacunae and in the cartilage matrix of the proliferative-upper hypertrophic zone, and they have been characterized as proteoglycan granules (19, 27). On the basis of histochemical, biochemical and morphologic observations, a degradation of these macromolecular proteoglycan complexes has been considered to be an integral part of the calcification process of the cartilage (see 10 for a review).

We conclude from our present findings that proteoglycan granule accumulation occurs in the growth plate cartilage and particularly in the narrow zone of calcification as a result of the presence of EHDP in the tissues. This accumulation can hardly be caused by an increased synthesis of matrix proteoglycans because there is biochemical evidence of an inhibition of synthesis by EHDP in

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treatment. Our conclusion is furthermore, based on the presence of a number of large membrane delimited vesicles in the epiphyseal/metaphyseal junction observed in the EHDP treated rats but not in the controls. The size and location of the vesicles are such that they cannot readily be characterized as a fraction of those matrix vesicles which are associated with the initial crystal formation. Lacking further proof we suggest that those particular vesicles may be lysosomal in nature, originating from the chondrocytes, and that their presence in the extracellular spaces in the area of capillary invasion indicates an abnormal lysosome function.

To sum up EHDP appears to have a direct inhibitory effect on initial crystal formation in the matrix vesicles. The inhibitory effects of EHDP in calcifying cartilage seem to require a continuous supply of EHDP since removal of EHDP treatment for four days resulted in resumed crystal formation. The degradation of proteoglycan complexes - an integrated part of the calcification process - is apparently inhibited by EHDP. A lack of degradation of the cartilage matrix, and of the hypertrophic chondrocytes as well may be of importance for the observed inhibition of capillary invasion. These changes may be due to a primary inhibitory effect on lysosomal function, as evidenced by an accumulation of lysosome like structures in the zone where calcification and erosion normally take place.

Financial support was obtained from the Swedish Medical Research Council (project No 24X-05005) and the King Gustaf V 80th Birthday Fund

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THE ANAL TRANSITIONAL ZONE

A Method for Macroscopic Demonstration

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Fenger C. The anal transitional zone. A method for macroscopic demonstration. Acta path. microbiol. scand. Sect. A, 86: 225-230, 1978

The anal canal extends from the upper to the lower border of the internal sphincter. The canal can histologically be divided into three zones according to the epithelial lining, being of colo-rectal type in the upper part and squamous in the lower part, while the middle part, where the epithelium varies, is called the anal transitional zone (ATZ). This zone can be demonstrated macroscopically using whole mount staining with Alcian dyes, which reveal it as light green or blue, contrasting to the dark stained rectal type mucosa above and the unstained squamous epithelium below. Comparison with histological sections shows that this zone corresponds with reasonable accuracy to the anal transitional zone.

Key words: Anal transitional zone, macroscopic demonstration.

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Received 3 x 77 Accepted 22 x 77

The epithelial lining of the anal canal is of colo-rectal type in the upper part and squamous in the lower part. Between these two, just proximal to the dentate line, is a zone in which the epithelium varies. This is called the anal transitional zone and has until now only been estimated on histological sections. The present paper deals with a method for macroscopic demonstration of this zone. By way of introduction a short survey on the nomenclature of the region will be given.

NOMENCLATURE

Gross Anatomy

The anal canal (Fig. 1) connects the rectum with the skin of the perianal region. The upper border (ano-rectal junction) is by anatomists and pathologists defined to be situated at the level of the pelvic floor, i.e. the upper border of the internal sphincter (7-12). Some clinicians, however, prefer to define the ano-rectal junction as taking place at the dentate line, calling the area above the dentate line per *anus* recti. The lower border is the anal opening which corresponds to the lower border of

the internal sphincter. Following the anatomical definition, as used in this study, the anal canal is about 3.5 cm in length (7-12).

The mucosal surface shows anal columns, anal sinuses and anal valves, terms which are all accepted in the international anatomical nomenclature (9). The line of the anal valves is referred to under a variety of names such as the dentate line, pectinate line, sinuous line, crypt line, valvular line, papillary line, ano-cutaneous line, mucocutaneous junction and ano-rectal line (7-18). As the term *dentate line* is used in as well the WHO-classification of intestinal tumours (13) and in *Morison's* textbook on gastrointestinal pathology (12) and as the configuration of the line more resembles a row of teeth than a comb, this term will be preferred in the following. The terms *Hulse's white line* (6) and *pecten* (17) seem ill defined and should be avoided (3).

Histology

The anal canal can be divided into three zones according to the epithelial lining, being of the colo-rectal type with crypts in the upper zone, of varying appearance above and at the level of the dentate line and squamous below. Further below the epithelium merges into the true skin of the perianal region.

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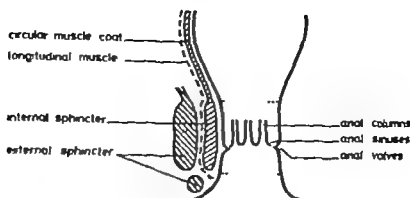


Fig. 1 Diagram of anal canal with nomenclature used

The middle zone was first described by *Robin and Cadial* in 1874 (16) although already *Bernard* in 1833 (1) noticed that the transition between the rectal mucosa and squamous epithelium was not sharp. In 1879 *Duret* (2) gave it the name zone moyenne or fibroide and from then on this area has been referred to under a variety of names based on as well embryological as macroscopic and histological observations, such as the cloacogenic zone, haemorrhoidal zone, membranous zone, intermediate zone, transitional zone, junctional zone and zona columnaris (5, 7, 18).

As the term transitional zone can be used for a wide variety of locations where epithelial types change (anal canal, cardia, cervix, uteri, salivary glands, bile ducts, etc.) this term is well understood. The term transitional or transepithelial epithelium is often used synonymous with urothelium and should therefore be avoided in this connection. In the following the term *anal transitional zone* (ATZ) will be used for that part of the anal mucosa that lies between typical uninterrupted rectal crypt bearing mucosa above and typical uninterrupted squamous epithelium below.

MACROSCOPIC DEMONSTRATION

In a previous paper (4) it was shown that the epithelium of the anal glands was characterized by a moderate mucus-production. As this epithelium in some respects resembles the epithelium of the ATZ, this led to the assumption that the ATZ could be demonstrated by whole-mount staining giving contrast to as well the strong mucus-producing crypt-bearing rectal mucosa above and the mucus free stratified squamous epithelium below. This method was introduced for stereo microscopic studies by *Landboe-Christensen & Parup* in 1970 (10) using PAS for stomach and duodenum and Alcian-green 3 BX (AG) or Alcian-blue 8 GX (AB) for colon and rectum.

The present paper will deal with a comparison between the macroscopic picture of the 3 different stains related to the conventional histological picture of the ATZ.

MATERIAL AND METHODS

The material comprises 18 anal canals obtained abdomino-perineal resection for rectal adenocarcinoma. In all cases the distance between the lowest border of the tumour and the dentate line was 4 cm or more. The specimens were suspended on a cork slab before frozen. The following procedure was performed, some specimens being prefixed in 10% buffered formalin:

- 1 Fixation in Little's AAF for at least 24 hours
- 2 Running water for about 10 min
- 3 Whole mount staining (see below)
- 4 Running water for about 1 min
- 5 Na_2CO_3 0.3% for 45 min
- 6 Running water for about 5 min
- 7 Storage in ethanol 70%

Before the whole mount staining the specimens were removed from the cork-slabs. The following stains were used:

- a Alcian-green 3 BX (Gurr) 0.5% in 1% acetic acid 40 min. at pH 2.5-3.0 (6 specimens)
- b Alcian-green 3 BX (Gurr) 0.5% in 1% acetic acid 120 min. at pH 2.5-3.0 (3 specimens)
- c Alcian-blue (Bio-rad) C 1 no. 74240 0.5% in 1% acetic acid 40 min. at pH 2.5-3.0 (5 specimens)
- d PAS (periodic acid) 0.5% 5 min. Schiff's reagent (E. Tomar) 5-30 min. (4 specimens)

Following the staining procedure, the specimens were replaced on cork slabs with a special designed plastic folio diagram of squares, each 2.5 x 0.4 cm and colour dias were produced with the camera in a standard position (Fig. 2).

The ATZ and its surroundings were then cut up along the lines on the diagram, resulting in 17-20 blocks of tissue. Each block was embedded in paraffin in a standard position with its left side upwards. The sections were stained with haematoxylin and eosin and examined under the light microscope. The borders of the ATZ were marked with ink-spots (Fig. 3).

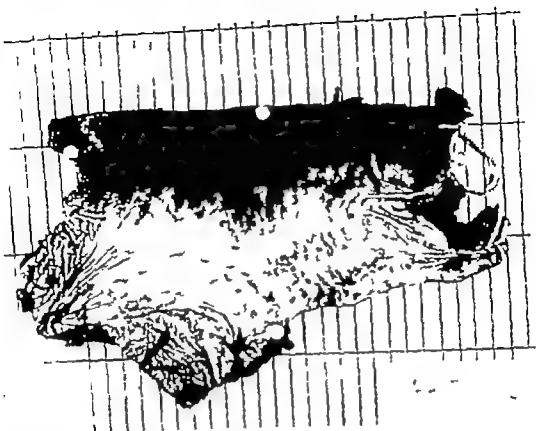


Fig. Anal canal stained with Alcian-green 3 BX for 40 min. Rather low position of ATZ



Fig. 1 Histological section from another anal canal. The ATZ is marked by ink spots. Part of the ATZ is located in an anal sinus, beneath which is seen an anal gland

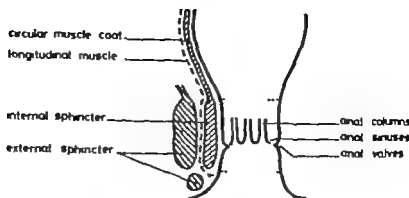


Fig 1 Diagram of anal canal with nomenclature used

The middle zone was first described by *Robin and Cadat* in 1874 (16), although already *Berard* in 1831 (1) noticed that the transition between the rectal mucosa and squamous epithelium was not sharp. In 1879 *Duret* (?) gave it the name zone moyenne or fibroide and from then on this area has been referred to under a variety of names based on as well embryological as macroscopic and histological observations such as the cloacogenic zone, haemorrhoidal zone, membranous zone, intermediate zone, transitional zone, junctional zone and zona columnaris (5, 7, 18).

As the term transitional zone can be used for a wide variety of locations, where epithelial types change (anal canal, cardia, cervix uteri, salivary glands, bile ducts etc.) this term is well understood. The term transitional or transitionocellular epithelium is often used synonymous with urothelium and should therefore be avoided in this connection. In the following the term *anal transitional zone* (ATZ) will be used for that part of the anal mucosa that lies between typical uninterrupted rectal crypt bearing mucosa above and typical uninterrupted squamous epithelium below.

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The present paper will deal with a comparison between the macroscopic picture of the 3 different stains related to the conventional histological picture of the ATZ.

MATERIAL AND METHODS

The material comprises 18 anal canals obtained by abdomino-perineal resection for rectal adenocarcinoma. In all cases the distance between the lowest border of the tumour and the dentate line was 4 cm or more. The specimens were suspended on a cork-slab before fixation. The following procedure was performed, some specimens being prefixed in 10% buffered formalin.

- 1 Fixation in Little's AAF for at least 24 hours
- 2 Running water for about 10 min
- 3 Whole mount staining (see below)
- 4 Running water for about 1 min
- 5 Na_2CO_3 0.3% for 45 min
- 6 Running water for about 5 min
- 7 Storage in ethanol 70%

Before the whole mount staining the specimens were removed from the cork-slab. The following stains were used:

- a Alcian-green 3 BX (Guir) 0.5% in 1% acetic acid 40 min at pH 2.5-3.0 (6 specimens)
- b Alcian-green 3 BX (Guir) 0.5% in 1% acetic acid 120 min at pH 2.5-3.0 (3 specimens)
- c Alcian-blue (Bio-rad) C 1 no. 74740 0.5% in 1% acetic acid 40 min at pH 2.5-3.0 (5 specimens)
- d PAS (periodic acid) 0.5% 5 min. Schiff's reagent (de Tomasi) 5-30 min (4 specimens)

Following the staining procedure, the specimens were replaced on cork-slabs with a special designed plastic folio diagram of squares, each 2.5×0.4 cm and colour data were produced with the camera in a standard position (Fig. 2).

The ATZ and its surroundings were then cut up along the lines on the diagram, resulting in 17-20 blocks of tissue. Each block was embedded in paraffin in a standard position with its left side upwards. The sections were stained with haematoxylin and eosin and examined under the light microscope. The borders of the ATZ were marked with ink-spots (Fig. 3).

3 mm from the light zone, mostly being smaller. In a few cases stained spots were found in the grey zone. These corresponded to traumatic lesions of the squamous epithelium. Mucust-producing epithelium was never found in this area.

DISCUSSION

The extent of the ATZ has until now been estimated on histological sections (5-14, 18). This method, however, only gives information on the extent of the ATZ in selected spots unless a tremendous number of sections are investigated. Using the whole mount staining method this is avoided and the zone can be estimated in its whole extent and related to macroscopic landmarks.

The fixative chosen is Little's AAF. This consists of ethanol added to formalin in order to give a better preservation of the surface structures and to this is added acetic acid, the action of which is to offset the shrinkage due to ethanol (11). AB can be used as well as AG but the last will be used in coming series because of reliability in delivery. AG 3 BX is used in a concentration of 0.5%. The staining period is prolonged to 40 min. when compared with other authors (10) because of the size of the specimens and in order to establish a better difference between cryptobioting and flat mucosa. Further extension of the period does not change the result.

Poststaining with sodium carbonate is known to convert AG & GX to monastral fast-blue, an insoluble pigment. Alcian-green 3 BX (and 2 GX) has been shown to contain a mixture of the phthalocyanine dye AB and the non-phthalocyanine dye Alcian-yellow (8). Poststaining with sodium carbonate is therefore likely to result in a considerable amount of monastral fast-blue in the AG coloured specimens, thus explaining the resemblance of colours in AB and AG stained specimens.

The pH level of 7.5 or slightly above is known to give a dark green (blue) colour in the presence of weakly sulfated mucosubstances and carboxyl groups of sialomucins (15).

In three fourths of the sections the ATZ corresponded to the light zone, but in the rest of the cases exact correlation could not be obtained. Possible explanations for this could be:

1. Reflexes in the photograph may be misinterpreted as light zone.
2. Deformation and shrinkage due to the embedding procedure may reduce the ATZ in histological sections.
3. Mucosa is not totally removed from the surface of the squamous epithelium.

4. The ATZ is sometimes covered by the anal valves.

Comparing the macroscopic with the histological picture one must also bear in mind that even a slight variation in the cutting up procedure together with a variation in the level from which the section is taken from the paraffin block may lead to a difference between the two. The difference of 1-2 mm seems to be within acceptable limits.

The above mentioned circumstances in connection with the fact that even careful suspension always leaves the mucosa slightly folded, and the fact that the ATZ is partly located in the anal sinuses makes planimetric studies too incorrect in two dimensional diagrams.

My thanks are due to the Departments of Pathology III Copenhagen, Høbrosbro and Aalborg for providing material for the study of the anal canal.

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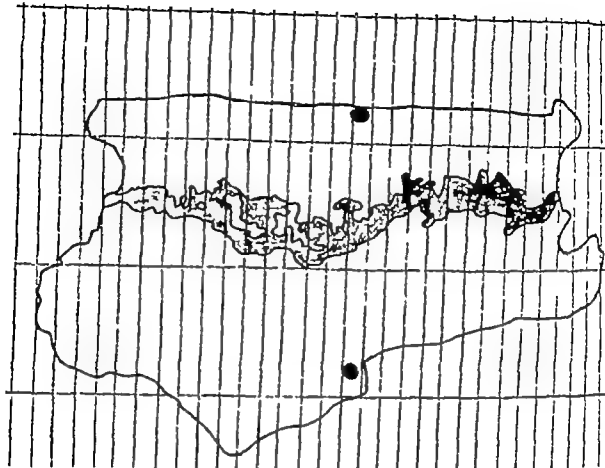


Fig 4 Paper diagram drawn after colour diapositive as shown in fig. 2. The white spots in the ATZ indicate where the colour is not typical.

The colour dias were projected on a paper with the same diagram as the plastic folio and the outlines of the specimen as well as the lines of colourshift were drawn on the paper diagram (Fig 4). The drawings were compared to the ink spots on the histological sections.

RESULTS

PAS Method

Macroscopic examination of the 4 specimens stained with PAS revealed a relatively sharp colourshift between magenta and grey at the level of the dentate line, without demonstrating the ATZ. The histological examination showed that only surface cells and free mucus were stained. The colourshift took place at the transition between the ATZ and the squamous epithelium independent of the length of the staining period.

AG and AB Methods

Macroscopic examination of the 9 AG and the 5 AB stained specimens showed immediately after the staining period in all cases 3 zones, a dark green (blue) zone above, a light green (blue) zone in the middle part just above the dentate line and a grey

zone beneath this line. After treatment with soda carbonate the differences in colour were less pronounced, AG as well as AB specimens taking rather turquoise colour.

The outlines of the light zone were mostly ragged at the upper border. The lower border was in all cases located at the dentate line, sometimes covering the luminal surface of the anal papillae. The proximal-distal extent of the zone varied in the series from a minimum of 1 mm to a maximum of 17 mm. Prolonging of the staining period from 4 to 120 minutes did not change the results.

The histological examination showed in all 1 specimens an ATZ lying between the rectal-type mucosa above and the stratified squamous epithelium below. Only surface cells and free mucus were stained. Comparison between the histological and macroscopic registration showed that the ATZ is three fourths of the sections corresponded to the light zone, although this correspondence sometimes could only be obtained if the slide was compared to a part of the light zone lying 1-2 mm to the right or left of the cutting line on the diagram. In the last one fourth of the sections the histological ATZ differed

METASTASIS SPREAD FROM SYNGENEIC MURINE TUMOURS

Establishment of a Test Protocol for Comparisons between Ascites Tumours and Their Progenitors.

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Hagmar B. & Ryd W. Metastasis spread from syngeneic murine tumours. Establishment of a test protocol for comparisons between ascites tumours and their progenitors. *Acta path. microbiol. scand. Sect. A, 86: 231-239, 1978.*

We have transformed two new VIC-induced tumours, a sarcoma (VICB1SS) and a carcinoma (VICB31SC) into ascites forms. When transplanted s.c. these ascites tumours grow as solid, quite undifferentiated tumours (AS = ascites solid tumours). We compared the metastasizability of the AS tumours with that of the parent tumours. In doing so, we used both the tail and the hind leg as transplantation sites. The tumours can be radically removed from both sites by amputations, which prolongs the survival time of the animals and permits metastases to grow into detectable sizes. As registered grossly and by microscopy, the AS tumours have a greater tendency of spread than the parent (SS/SC) tumours. VICB-21AS grows quicker than 21SS and gives rise to more lymph node metastases. When transplanted to the tail the AS tumour also gives a higher incidence of lung metastases. We detected no such difference by leg-transplanted tumours. VICB31SC did not produce any detectable metastases at all, while 31AS, particularly from the tail, gave rise to numerous lymph node and lung metastases. There were no differences in tumour size or growth rate to account for this difference. Thus ascites conversion has changed the carcinoma VICB31SC into an undifferentiated, metastasizing tumour as detected by our procedure. The design of test protocols to detect metastasizability is discussed.

Key words: Murine tumour, syngeneic, metastases, ascites tumours.

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Received 5/77 Accepted 23 xii 77

Factors governing the metastatic spread of malignant tumours are still largely unknown. In spite of efforts to elucidate them (Kerohom *et al.* 1959; Clifton & Agreston 1961; Rosenblatt *et al.* 1961; Nafise 1962; Rosso *in al.* 1967), we also lack generally-accepted models to compare the metastasizability of different experimental tumours.

One field where such experimental models would be needed is the comparison of ascites transformed tumours with their solid progenitors. In a murine system (Hagmar 1974 a, b, c) ascites conversion altered the spontaneous metastasizability and the

metastasis pattern of iv. infused sarcoma cells. These findings prompted speculations about the importance of cell-surface characteristics for ascites transformation, i.e. metastasis formation.

In the present study we apply a new test protocol to comparisons between two newly-induced murine syngeneic tumours, a sarcoma and a carcinoma, and their corresponding solid ascites forms.

The tumours were transplanted as solid tumour pieces to the tail or to a hind leg, where they could be amputated at different times after transplantation.

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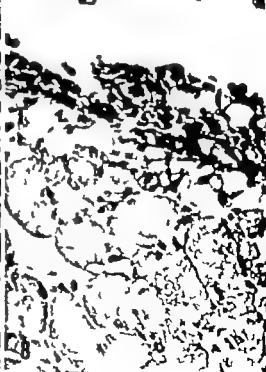
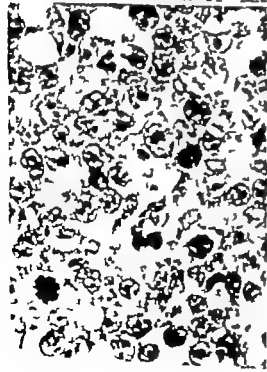
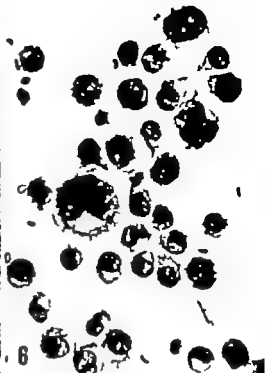
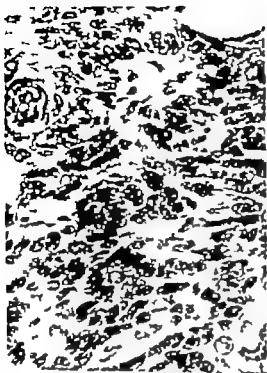
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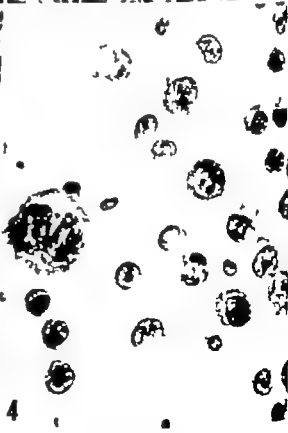
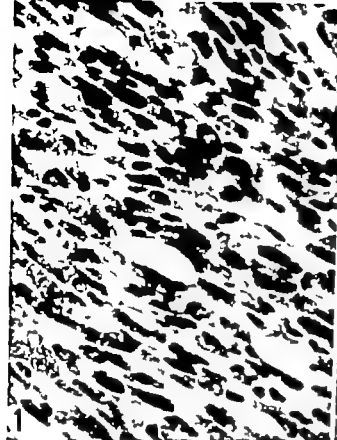
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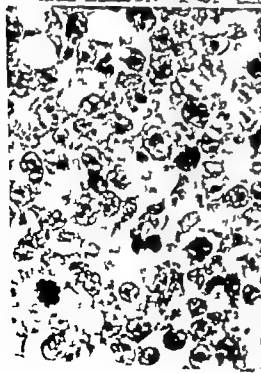
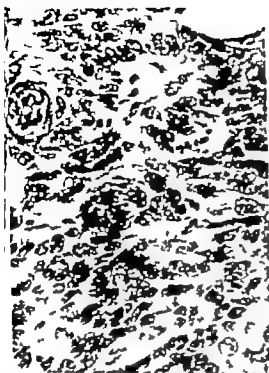
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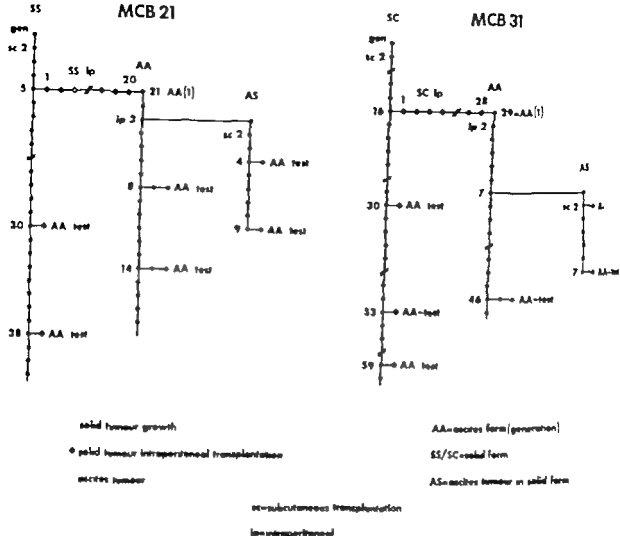


Fig 9 Schematic illustration of the transformation of MCB21 SS and MCB31 SC to the ascites tumours MCB21 AA and MCB31 AA

Sc injection of 0.1 ml undiluted 21 or 31 AA gave rise to solid tumours (AS-tumours). When these AS-tumours are re-transplanted i.p. as mice or solid pieces (indicated by AA-test) the tumours grow as ascites tumours, i.e. as a suspension with high cellularity and with little infiltration in the abdominal wall. I.p. transplantation of mice or solid pieces of 21-SS and 31-SC causes extensive tumour infiltration in the abdominal wall and gives rise to little ascites.

Fig 1 MCB21 SS gen 1 A differentiated fibrosarcoma. Note the spindle-cell structure (X 1850)

Fig 2 MCB21 SS gen 30 The tumour is less well differentiated and the cells more polymorphous. The tumour has retained its character of a fibrosarcoma, however (X 1850)

Fig 3 MCB21 AS from AA gen 37 The tumour is completely undifferentiated (X 1850)

Fig 4 MCB21 AA gen 5 The tumour cells generally are small with scanty cytoplasm. Near the left margin of the picture is a binuclear giant cell. Note the scarcity of red blood cells (X 1850. Papaniolaou)

Fig 5 MCB31 SC in gen 38 growing in the peritoneal wall after i.p. transplantation in one of the AA tests. Note that the tumour is still a rather well-differentiated squamous cell carcinoma with horn formation (upper left) (X 1850)

Fig 6 MCB31 AA in gen 15 The cells generally are larger than 21 AA cells with more cytoplasm. In the center a giant cell with three nuclei (X 1850. Papaniolaou)

Fig 7 MCB31 AS from AA gen 4 Like 21 AS, the tumour is quite anaplastic and rather polymorphous. A mitosis at the lower left (X 1850)

Fig 8 MCB31 AS from AA gen 14 The tumour is growing in the peritoneal wall after transplantation of AA cells i.p. Note the poor penetration of the underlying muscle and compare with Fig 5 (X 1150)

MATERIAL AND METHODS

The tumours were induced in inbred CBA mice by gastric feeding of 20 mg 20-methylcholanthrene (MC) given in 10 doses, 2 mg/dose, twice weekly.

MCB 1 arose subcutaneously in a female mouse 4.5 months after termination of the MC-feeding. It is a fibrosarcoma that has become less differentiated during serial transplantation. It has retained its character of a spindle cell fibrosarcoma, however (Figs 1 and 2). It is subsequently called MCB 1 SS (solid sarcoma).

MCB 11 arose in the skin of a male mouse 3 months after induction. The tumour is a well-differentiated squamous cell carcinoma with horn formation (Fig. 3). This tumour is called MCB 11 SC (solid carcinoma).

Acute transformation. We followed the procedure described by Klein (1951) and his criteria for acute conversion. The procedure with serial transplantation of tumour tissue and six incisions to the abdominal cavity is illustrated in Fig. 9. As can be seen, the first generation of the acute form of MCB 21 (MCB 21 AA) corresponds to SS generation III. Similarly MCB 31 AA corresponds to its first generation to SS gen. 31. Several of the AA tumours are shown in Figs. 4 and 6.

When transplanted s.c. both acute tumours grow as

solid tumours (AS = ankytic solid form). Both AS tumours are quite undifferentiated and extremely cellular (Figs. 3 and 7). The tumours in the present experiments were taken from serially-transplanted animals. We used as far as possible SS and AS tumours in comparable generations (Tables 1-3). The AS tumours were obtained by s.c. injection of ankytes to donor mice, one generation before the experiment. The tumours were transplanted by sterilized trocars, 3 pieces $\approx 1 \text{ mm}^3$ to the hind foot (cf. Hagmar 1974 c) or s.c. onto the tail (Hagmar and Boerid 1968). In one experiment we transplanted MCB 21 SS similarly i.m. into a hind leg. The tumours were removed by amputation of the whole hind leg (Hagmar 1974 c) or the tail (Hagmar and Boerid 1968) under ether anaesthesia. The leg tumours were weighed with the leg, the tail-tumours with 2 cm of the tail.

The animals were observed until spontaneous death. Surviving animals were killed after 6 months. All animals were subjected to a complete autopsy with registration of any gross tumour. The lungs, liver and any doubtful tumour or lymph node were fixed in formalin. The lungs were weighed after fixation, before being processed for histological examination. The serial-sectioning technique described by Boerid (1965) was used on lungs and livers.

TABLE 1 MCB 21 SS Spontaneous Metastases from Tumours Transplanted Subcutaneously (s.c.) on the Tail or into Hind Leg or Intramuscularly (i.m.) into a Hind Leg. Each Group Comprised 8 Mice for Experiments in Tumour Gen. 14, 10 Mice for Gen. 13 and 52

recpt no	Site	Time on day after transpl	Median survival time (days)	Average tumour weight (g)	Lungs		Extrapulmonary metastases	
					Incidence of metast.	Average weight (g)	Lymph nodes metastases	Other sites incidence (number and site)
4	Tail	14		0.20	0/8	0.19	0/8	0/8
		21	60	0.35	2/7 ¹	0.44	0/7	0/7
		3	56	1.33	7/7 ²	0.64	3/7	0/7
5	Tail	14	62	0.18	1/10	0.23	0/10	0/10
		21	53	0.25	1/10	0.16	1/10	0/10
		23	55	0.51	5/10	0.31	4/10	0/10
14	Leg	14	53	1.18	1/8	0.28	0/8	0/8
		21	53	2.06	8/8	0.51	0/8	0/8
		23	47	5.40	6/6 ³	0.74	0/6	0/6
13	Leg c	14	113	0.76	1/10	0.24	0/10	0
		17	54	0.95	5/9 ²	0.37	0/9	2/9 (1 kidney 1 str. artery)
		1	72	1.29	5/8 ²	0.48	0/8	1/8 (1 ovary)
14	Leg m	14	88	0.7	7/8	0.68	0/8	0/8
		1	46	1.90	7/7 ²	0.59	0/7	0/7
		23	44	6.59	3/3 ³	0.71	2/3	1/3 (1 adrenal)

¹ Animals dead of unknown cause
² Animals lost by operation mortality
³ Animals discarded because of tumour recurrence

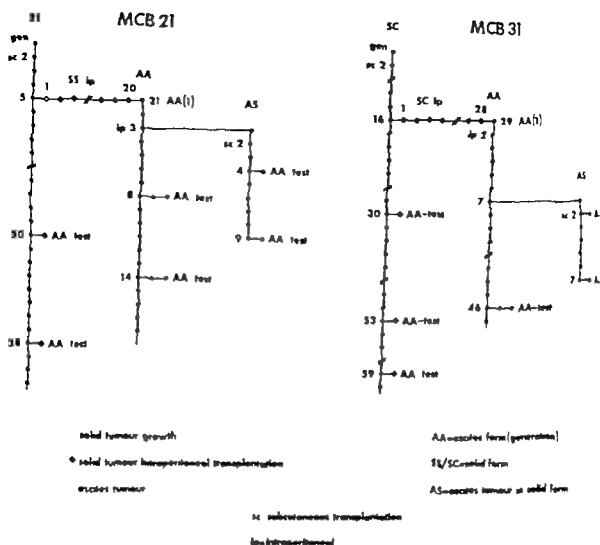


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Fig 7 MCB31 AS from AA gen 4 Like 21 AS, the tumour is quite anaplastic and rather polymorphous. Mitotic at the lower left (X 1850).

Fig 8 MCB31 AS from AA gen 14 The tumour is growing in the peritoneal wall after transplantation of AA cells i.p. Note the poor penetration of the underlying muscle and compare with Fig 5 (X 1150).

TABLE 3 MCB31-SC and AS Spontaneous Metastases from Tumours Transplanted s.c. on the Tail or Into a Hind Leg

		Lungs					
Transpl site	Days after transpl	Median survival time (days)	Average tumour weight (g)	Focus of metast.	Average weight (g)	Lymph nodes incidence	Other sites incidence
MCB31 SC (8 mice per group in tail-transplanted mice, 10 mice in leg-transplanted)							
8 Tail s.c.	14		0.23	0/5 ⁴⁾		0/5	0/5
	21		0.31	0/8		0/8	0/8
	28		0.49	0/7 ²⁾		0/7	0/7
78 Leg s.c.	14		1.09	0/7 ⁴⁾		0/7	0/7
	1		1.29	0/9 ²⁾		0/9	0/9
	28		1.50	0/10		0/10	0/10
MCB31 AS (8 mice per group in gen. 4, 15 mice in gen. 26)							
AA gen.							
6 Tail c	11	39	0.14	0/8 ⁴⁾	0.16	1/8	0/8
	20	43	0.18	6/14 ¹⁾	0.15	1/14	0/14
	29	41	0.29	7/13 ^{2a)}	0.16	13/14	0/13
4 Tail s.c.	8		0.17	0/7 ¹⁾	0.25	0/7	0/7
	16	37	0.4	3/8	0.21	5/8	0/8
	23	38	0.3	1/7 ¹⁾	0.2	1/7	0/7
4 Leg c	8		0.78	0/6 ⁴⁾	0.24	0/10	0/10
	16		1.04	0/5 ^{2a)}	0.24	0/5	0/5
	3	38	2.40	2/4 ²⁾	0.18	2/4	0/4

1) Animal(s) dead of unknown cause

2) Animal(s) lost by operation mortality

3) Animal(s) discarded because of tumour recurrence

4) Group reduced because no tumour was found histologically at implantation site

DISCUSSION

Too little is yet known about the metastasis process to attempt to develop specific anti-metastatic therapy. It is then of course highly desirable to develop experimental systems in which different phases of metastasis can be studied. Old, often allograftable tumours have been widely used for this purpose (cf. *Wefgren et al.* 1966) probably because newly-induced tumours are said to give rise to few if any metastases (*Nielsen* 1962, *Kihl* 1971, *Currie* 1974). New tumours in syngeneic systems must be preferable however because of their closer similarity to autogenous tumours, such as those in humans. We think that the lack of metastases from new tumours is largely due to a deficient screening technique for metastasizability. In our laboratory we have accordingly devoted part of our metastasis

studies to develop new metastasizing tumours in syngeneic murine systems (*Wefgren et al.* 1966, *Hagmar* 1974 a-c).

When screening new tumours for spontaneous metastasizability we think that the screening tests must fulfil certain criteria in order to be relevant.

1/ The tumours must be transplanted as solid pieces. Otherwise, when tumour cell suspensions are injected s.c. or i.m. tumour cells may disseminate directly by lymph or blood vessels and give a false impression of a spontaneous spread (*Nielsen* 1962, *Fidler et al.* 1969).

2/ The tumours must be radically resectable. If not, the animals will succumb to their primaries, before metastases have grown to detectable sizes (*Olsenfeld* 1941, *Nielsen* 1962, *Händler et al.* 1964).

Differences in incidences, survival time, lung and tumour weights were compared by the Fisher permutation test. Differences with $p < 0.05$ were considered significant.

RESULTS

MCB 21 The results are shown in Tables 1 and 2. The tumours grew progressively in all sites.

MCB 21-SS tumours in the leg grew quicker intramuscularly than subcutaneously and in most animals the i.m. tumours could not be radically resected after 4 weeks. Therefore and because the tumours generally ulcerated the i.m. transplantation site was abandoned and not used for comparison with 21 AS. However the i.m. SS tumours started to metastasize to the lungs before the s.c. SS tumours (2 weeks $p < 0.005$).

Lung metastases were more frequent from s.c. tumours in the leg than on the tail. On the other hand SS tail tumours tended to give rise to more lymph node metastases than SS leg tumours (4 weeks $p < 0.10$). The results were similar in different SS generations.

MCB 21 AS grew quicker in the leg than on the tail. The quick growth prevented us from using the 4 weeks' growth period for leg tumours. The incidence of lymph node metastases tended to be higher from the tail-tumours, however than from the larger leg tumours.

When we compare 21 SS with 21 AS we restrict the comparisons to SS gen. 33 AA AS gen.

14 (leg s.c.) and SS gen. 52 AA AS gen. 21 (tail) because these generations correspond roughly to the number of *in vivo* transfers.

The AS tumours grew quicker than the SS tumours. The difference is significant for both tumours ($p < 0.0075$) and tail tumours ($p < 0.01$). Lymph node metastases were more frequent from AS than from SS. For tail tumours the differences are significant, where the times for appearance were similar (21 days $p < 0.02$, 78 days $p < 0.01$). The incidences of lung metastases were similar for SS and AS leg tumours, but AS tail tumours give rise to lung metastases in more mice than SS. tumours did (3 weeks $p < 0.02$, 4 weeks $p < 0.01$).

Extrapulmonary metastases other than to lymph nodes, were rare for SS and AS alike, regardless of the site of transplantation.

MCB 31 (Table 3) The SC tumour grew well in the tail and in the leg. The growth was very much quicker in the latter site. No gross or microscopic metastases were detected.

The 31 AS tumour failed to grow in some mice, especially on the tail and in generation 76. Of the transplanted mice, only 2 had metastases in the lungs. Tail-transplants, on the other hand, gave rise to numerous metastases in the lungs and to regional (tail root) lymph nodes. From gen. 76 all mice except two carrying their tumours for 70 days or more, had lymph node metastases. About half the number of these mice also had lung metastases. Except in lymph nodes, no extrapulmonary metastases were found.

TABLE 2 MCB21 AS Spontaneous Metastases from Tumours Transplanted Subcutaneously (s.c.) on the Tail or on the Hind Leg. Each Group Comprised 8 Mice for Experiments in Tumour Gen. 14 and 15 Mice for Gen. 23

Transpl. gen. (AA)	Site	Days after transpl.	Median survival time (days)	Average tumour weight (g)	Incidence of metast.	Lungs		Extrapulmonary metastases	
						Average weight (g)	Lymph nodes incidence	Other sites incidence (number and out of 8)	
14	Leg s.c.	14		1.06	0/8 ⁽¹⁾	0.20	0/8	0/8	
		17	47	1.52	2/6 ²⁾	0.24	7/6	0/6	
		21	49	2.15	3/8	0.30	2/8	1/8	
23	Tail s.c.	16	54	0.19	3/12 ⁽¹⁾	0.27	5/13	0/15	
		21	50	0.37	10/14 ⁽²⁾	0.77	9/13	0/15	
		28	49	0.57	11/13 ⁽¹⁾⁽³⁾	0.25	17/14	1/14	(1 orbital)

1) Animal(s) lost by operation mortality.

2) Animal(s) discarded because of tumour recurrence.

3) Animal(s) discarded because of cannibalism.

4) Group reduced because no tumour was found histologically at implantation site.

We thank Miss Marianne Brønner and Miss Agnete Jacobsen for skilful technical assistance, and Mrs Astrid Bjørnshede for typing the manuscript.
The study is supported by grants from the S. Danish Cancer Society (No. 645-875-06X).

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iii/ The transplantation mode should permit detection of both lymphatic and haematogenous spread. This criterion which is fulfilled by leg and tail transplantation has the disadvantage that it may be impossible to separate one route of spread from the other. This may call for variations, where one way of spread can be studied separately (Rovso *et al* 1967 Carr & McGlinn 1973 Hewitt & Blake 1977).

iv/ Metastases must be looked for and registered not only grossly but also by microscopy. There is of course, a limit to how extensively tissues can be processed for histology. We think that at least the primary target organ such as the lungs for tumours drained by the caval veins, should be investigated preferably by serial sectioning (Boernd 1965 Mellgren 1976).

Our present experiments follow a test protocol that fulfils these criteria and which we think will reveal truly metastasizing tumours. We included two resectable tumour sites, the leg or foot and the tail because Nielsen (1962) in his review showed that the tail for some tumours is a more favourable site for metastases to develop than subcutis elsewhere. MCB 31 AS developed more metastases from the tail than from the leg, especially in generation 26 (Table 3). In generation 4 the tumours grew poorly on the tail as seen from the low tumour weights which may explain the lower incidence of metastases from this generation of 31 AS. The poor growth-conditions on the tail are presumably also responsible for growth failures which reduced some of the groups (Table 2 and 3). This is a finding we have met with several times in the past and a reason why one probably should not use the tail as the only transplantation site in metastasis screening tests.

Our reason for testing the present tumours was to see whether ascites conversion would change the metastatic behaviour of two new tumours. We chose one tumour of mesenchymal origin the fibrosarcoma MCB 21 and an epithelial tumour the squamous cell carcinoma MCB 31.

MCB 21 AS tumours gave rise to more lymph node and lung metastases from the tail than the corresponding SS tumours. Possibly the tail transplantation here permitted us to detect a greater tendency of spread for AS than for SS.

MCB 31 showed a much more distinct difference between the SC and AS forms than MCB 21. While 31-SC did not give rise to any detectable metastases at all, AS developed numerous lymph node and lung metastases, particularly from the tail. There were no differences in tumour size to explain this finding. Thus, the ascites transformation converted MCB 31

from a non-metastasizing to a metastasizing tumour under our experimental conditions.

Our present findings can be compared with change in metastasizability encountered at SC conversion of the fibrosarcoma MCB101 (Hagmar 1974 a-c). From this tumour both the SS and AS forms give rise to metastases. But the pattern of metastasis changed from being mainly in the liver for the SS tumour to being mainly extraportal for the AS tumour.

We speculated then about possible causes of this change, which might be related to the SC transformation, and suggested that surface modifications on the AS tumour cells were involved. This is our main hypothesis also for MCB 31 for there is no difference in «malignancy» between the SC and AS tumours to explain the altered tendency of spread as shown by transplantation tests (Rid & Hagmar 1978 a).

In fact, it is confusing that MCB 21 and 31 give rise to blood-borne metastases at all, for both tumours require large cell doses (10^4 - 10^6 cells) transplantation *iv* (21 SS is not even transplantable with 10^6 cells (Rid & Hagmar 1978 a). The pattern of blood-borne spread, however with almost exclusively lung metastases, is common for spontaneous spread in the present experiments: *iv* induced artificial «metastases» (Rid & Hagmar 1978a).

What cell changes may then take place at SC conversion that affect the metastasizability of tumours? As mentioned we suspect that surface alterations are involved. They would permit the cells to divide and thrive uncellularly in ascites on the one hand (cf Klein E 1955 Pardee 1971) and facilitate cell separation and spread on the other hand, when growing in solid *s.c.* transplants.

In preliminary experiments we have shown that surface differences exist between the SS and AS forms, e.g. in lectin agglutinability between 31 SC and AS (Rid & Hagmar 1978 a). The ultrastructure of the ascites tumour cells is also different from that of solid tumour cells (Rid & Hagmar 1978 b). As with some other methods of study such as cytochemistry and cell electrophoresis (Rid & Hagmar 1978 a) the data are still difficult to evaluate, however because of artifacts on the cells produced by enzyme treatment required to bring cells from the solid tumours into suspension.

Further studies are obviously needed on the causes of the increased metastasis spread by our AS tumours, particularly 31 AS compared to the SS tumours. The present study shows, however that the new MCB tumours may be of use as experimental models of the spontaneous metastasis process.

HEART AUTOPSY IN ISCHEMIC HEART DISEASE

An Autopsy Protocol

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Hansen, Birgit Fischer: Heart autopsy in ischemic heart disease. An autopsy protocol. *Acta path. microbiol. scand. Sect. A*, 86: 241-44, 1978.

The commonly performed autopsy of the heart often fails to demonstrate a satisfactory clinicopathological correlation in cases of death due to ischemic heart disease. An autopsy protocol devised specifically for the study of hearts from patients succumbing to ischemic heart disease is presented. This protocol has been employed in a consecutive series of 63 patients with ischemic heart disease dying at the medical department B, Rigshospitalet in the period 1 September 1975 to 1 May 1976. It comprises a meticulous histological examination of the coronary arteries, including quantitation of intimal narrowing. The ventricular myocardium is sliced transversely and the Nitro-BT test is applied to identify and delineate acute myocardial infarction, the extent of which is quantitated by point-counting. In a search for histopathological changes responsible for conducting disturbances, the sinoatrial and the atrioventricular nodes are examined histologically.

Key words: Heart autopsy, ischemic heart disease, nitro-BT test, quantitation.

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Received 25.11.77

Accepted 24.12.77

Ischemic heart disease is a major public health problem, and the mortality in Denmark, as elsewhere in the Western World, is steadily increasing. In Denmark it is about 16 000 per year.

During the last decades there has been an increasing call for better heart autopsy, especially concerning the early diagnosis of acute myocardial infarction (AMI). In autopsies as a whole the aim is a demonstration of clinicopathological correlations, and a routine autopsy technique should ideally be modified according to the object of the examination.

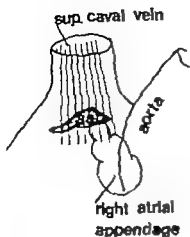
The autopsy protocol presented here is specifically devised for the study of ischemic heart disease and has been employed in a consecutive series of 63 patients dying at the medical department B, Rigshospitalet, Copenhagen in the period 1 September 1975 to 1 May 1976. A clinical diagnosis of AMI was, according to the criteria of WHO (18), in 41 patients definite and in 17 patients possible. In 8 cases of sudden death there were insufficient data for diagnosis, but they were considered as sudden cardiac death.

METHOD

The heart is removed, including intact aorta. Weight, outer measurements and visible changes in the pericardium are recorded, and the heart is placed in a deep-freezer for 30 minutes. The (ventricular) myocardium is sliced transversely in 1 cm thick slices to within approximately 1 cm of the aortoventricular groove. The myocardial slices are rinsed in running cold water and are examined to identify AMI: infarction, fibrosis, aneurysms, ruptures, changes in endocardium and mural thrombi. All findings are recorded on schematic drawings of myocardial slices (Fig. 1). The nitro-BT test (10) is performed on all slices (Fig. 2) and the result is recorded on similar schemes. All slices are then transferred to 10 per cent buffered formalin.

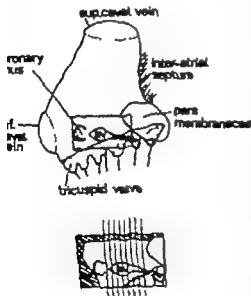
In the aortic root the coronary orifices are examined, and possible changes are recorded. The left atrium is opened and the mitral orifice is examined from here and from the ventricular aspect. The right atrium is, in preparation for subsequent examination of the sinoatrial and atrioventricular nodes, lightly packed with cotton wool. The heart cord specimen is then transferred to formalin.

Quantitation of the AMI is done by point-counting (9, 13), and to avoid shrinkage this is done within 24 h.



Opened right atrium anterior aspect

4 Schematic drawing showing the localization of the atrial node (an) & crucial lines indicating how strips are cut for pathological examination



Opened right atrium, anterior aspect

Fig. 4 Schematic drawing showing the localization of the atrial node (an) & of the anterior wall of the right atrium. Vertical lines indicate how strips are cut for pathological examination from the primarily isolated

as described by *Hudson* 1963 (6) (Fig. 4 and 5). They are sectioned in 5 mm blocks, and in each block serial sectioning is performed in levels at 2 mm intervals. In each level with 6 sections for immediate staining (hematoxylin and eosin, Verhoeff elastic-Van Gieson and diastase silver) and 11 sections for additional stain (as above). AMI infarction, fibrosis and pathological changes in intramural parts of the supplying arteries are recorded.

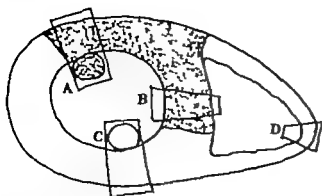
DISCUSSION

AMI is generally considered to be the result of thrombotic occlusion of the coronary arteries. A great number of non-thrombotic AMIs have been reported, however, and other pathogenic mechanisms than thrombosis seem at times to be responsible for the development of an AMI.

Transverse sectioning of the coronary arteries has been performed in innumerable investigations. This technique seems ideal in cases of ischemic heart disease if the object of the examination is to visualize not only recent occlusions but, just as essential, the degree and localization of arterial stenosis. Blood flow through a coronary artery is not decreased until at least 75 per cent of the lumen is obliterated (14), and quantitation of terminal narrowing is thus essential in separating cases with normal from cases with abnormal coronary flow.

The coronary arteries may for a shorter or longer course of their epicardial run disappear under the most superficial layers of myocardium. For this phenomenon *Gelfinger* 1951 (5) proposed the term "the mural coronary" and he found that mural arteries were rarely affected by arteriosclerosis. *Polder & Krulink* 1961 (11) found that the pre-mural segments of coronary arteries were sites of predilection for sclerotic processes and that coronary thromboses most frequently was seen here - suggesting that myocardium covering might play a role in the pathogenesis of coronary thromboses.

The advantages of transverse slicing of the ventricular myocardium are many. Changes in both the left and the right ventricular myocardium are visualized in many levels, and it is possible to estimate the exact extent of an AMI both circumferentially (anterior, posterior or septal AMIs) and according to what layers of the ventricular wall are involved (transmural, subendocardial or combined AMIs). Small AMIs of the subendocardial type are revealed by transverse slicing (1), whereas flat-cutting of the myocardium, when performed as intended through the middle of the wall, fails to reveal such AMIs. Isolated AMIs of the papillary muscles are also demonstrated by transverse slicing (3). Last, but not least, this technique is much easier to perform, especially in



Schematic drawing of myocardial slice

Fig 1 Schematic drawing of myocardial slice showing left ventricular antero-septal AMI involving the anterior papillary muscle. A, B, C and D indicate how samples of myocardium are taken systematically for histological examination

fixation in formalin. Each of the nitro-BT tested myocardial slices is covered by a transparent cellulose film on which is drawn a regular net of quadrants measuring 2×2 mm. Total area of left ventricular myocardial mass including the interventricular septum is counted first, area of AMI next, and the result is recorded in per cent AMI. In cases of right ventricular

Imbedding in paraffin Decalcification (Calabre) is done prior to or following the cross-sectioning, depending on the degree of calcification. Right or left coronary artery dominance is estimated and a record is made of which segments of main arteries feed important branches, i.e. ramus margo acutus from the right coronary diagonales from the left anterior descending and ramus margo obtusus from the left circumflex. Minor branches are also serially sectioned, serially sectioned and decalcified before imbedding in paraffin. Arteries are sectioned to an outer diameter of 2-3 mm. When ventricular myocardium is sliced as described, the left and the left circumflex arteries are found on the left cordis specimen until either one of the dominant arteries continues as a posterior descending artery. The left coronary artery and the first 2-4 cm of the anterior descending artery are also found on the basis cordis specimen. The further run of arteries is isolated from the myocardial slices. Each arterial segment is serially sectioned at levels at 2.5 mm intervals, in each level with 4 tests for immediate staining (hematoxylin and eosin, Verhoeff elastic, Van Gieson) and 4 for additional staining (Lendrum's fibro-stain, phosphotungstic acid, hematoxylin). Acute and old occlusive lesions are recorded and luminal narrowing is recorded in 4 per cent less than 75 per cent, 25-50 per cent, 50-75 per cent, 75 per cent or more (14).

Samples of myocardium for histological examination are taken systematically (see Fig 1), from the border of AMI as revealed by the nitro-BT test, from regions and from localized changes in endo- and pericardial. The stains are hematoxylin and eosin and Verhoeff elastic, Van Gieson.

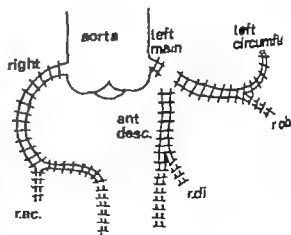
The sinoatrial and atrioventricular nodes are held



Fig 2 Myocardial slice after the nitro-BT test showing a localized subendocardial AMI in the posterior wall of the left ventricle involving the posterior papillary muscle

AMI quantitation is performed on the free right ventricular wall.

After 7 days fixation of the basis cordis specimen in formalin the epicardial parts of the coronary arteries are excised unopened with surrounding epicardial fat and little underlying myocardium. The arteries are cross-sectioned at 6-8 mm intervals (Fig 3); the segments are serially numbered and the proximal face of each segment is marked with Indian ink as a guide to correct



Cross-sectioning of coronary arteries

Fig 3 Schematic drawing showing cross-sectioning of coronary arteries. Peripheral segments of the arteries (stippled segments) are isolated from the myocardial slices. r.a.c. ramus margo acutus, r.d. ramus diagonales, r.ob. ramus margo obtusus.

RENAL INFECTION AFTER ILEAL CONDUIT URINARY DIVERSION

An Autopsy Study

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Bergman B & Knutsson P. Renal infection after ileal conduit urinary diversion. An autopsy study.
Acta path. microbiol. scand. Sect. A, 86: 245-250, 1978

Autopsy records from cases with urinary bladder carcinoma, consisting of patients operated upon with conduit urinary diversion and controls without diversion, were studied. We found macroscopical evidence of upper urinary tract infection (renal abscesses and/or pus-filled pelvis/ureters and/or reddish mucosa in pelvis/ureters) in 18 of 21 conduit cases without evidence of cancer at autopsy and in 14 of 50 control cases. The difference is statistically significant ($p < 0.0005$). Abscesses were found more often in conduit cases (10/21) than in non-diverted cases (8/50) ($p < 0.01$). The known connection between dilated ureters and/or pelvis and urinary tract infection was demonstrated in non-diverted cases.

Key words: Ileal conduit urinary diversion, urinary tract infection, pyelonephritis, renal abscess, autopsy.

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Received 9/78 Accepted 27/78

The importance of bacteremia in the genesis of renal damage has been intensively discussed. Roentgenologically demonstrable acquired renal lesions were shown in 7 per cent of children with urinary tract infection by Winberg *et al* (15). They later reported that some of these 7 per cent with renal lesions developed reduced renal function (6). However, other studies have failed to confirm relations between bacteremia and renal damage (1, 9).

In an autopsy study McDonald *et al* reported a significant relation between pyelonephritis and bacterial count in the post mortem urinary specimen (11). Pyelonephritis was found in 20 per cent of the cases, and in 6 per cent the lesions were so extensive as to be the major immediate cause of death. In another 6 per cent the lesion was considered to have been a contributory cause of death.

If there is a correlation between bacteremia and

renal damage it would imply that groups of patients with a high frequency of bacteremia also have a high frequency of renal damage and/or signs of upper urinary tract infection at autopsy.

Paraplegic patients constitute such a group with a high frequency of bacteremia (8). In earlier autopsy studies of paraplegic cases uremia was found to be the cause of death in 38-65 per cent (1, 10). In 1948 Dietrick & Rens found renal diseases in 20 per cent of paraplegic cases at autopsy. 11 per cent of the deaths were attributed to acute and/or chronic pyelonephritis (3).

The aim of this study was to investigate whether the high frequency of bacteremia in patients with urinary conduit diversions (5, 12) was similarly reflected in a high frequency of serious infection in the upper urinary tract at autopsy.

Urinary tract infection may be transmitted in the urological department and therefore we used as controls non-diverted cases followed with the same intervals as the diverted cases.

cases with infarction fibrosis. In the WHO report 1970 (17) on the pathological diagnosis of ischemic heart disease, transverse slicing is recommended as the method of choice, and the same conclusion is found in a proposed autopsy protocol by Schwartz *et al* 1975 (15). In this protocol transverse slicing is conditional for application of the nitro-BT test and subsequent quantitation of the AMI.

The pathological diagnosis of AMI is based on naked-eye findings, the nitro-BT test and histological examination. Changes in the myocardium visible to the naked eye cannot be demonstrated until after 15 hours, and the primary changes are non-specific: pallor, oedema and a slight change in consistency (8, 17). The possibility of establishing an early pathoanatomic diagnosis of AMI through demonstration of loss of dehydrogenases in the infarcted area (nitro-BT test) was first described by Nachlas & Shnitka 1963 (10). The nitro-BT test, however, is also time limited since AMIs that have persisted for less than 6–8 hours will not be revealed (1, 10, 12). False positive reactions have not been demonstrated, but false negative reactions are occasionally seen, especially in big AMIs and in cases with myocardial rupture. It has been suggested by us (1) that poor drainage from the infarcted area might be the explanation. Autolysis has no influence on the nitro-BT test when performed within 72 hours after death and provided that the body was kept at 4° Celsius (4). The earliest definite histological sign of AMI is infiltration with granulocytes, and at 24 hours these will be present in considerable numbers at the edges of the AMI (8, 17). Histological examination of AMIs only revealed by the nitro-BT test thus might be negative, although non-specific and qualitative changes can be demonstrated at the borderline of AMI (1).

Quantitation of AMI is done by point-counting and the accuracy is equal to that of planimetry (9, 13). It demands a clear-cut delineation of the AMI and only AMIs of nodular type and those seen in relation to infarction fibrosis are technically difficult.

Continuous electrocardiographic monitoring of patients with AMI has shown a high incidence of arrhythmias (7). Histological examination of the sinoatrial and atrioventricular nodes is often disappointing (16) since acute lesions are seldom demonstrated. The examination performed should reveal even small AMIs, infarction fibrosis and pathological changes in intranodal parts of the supplying arteries.

The study was supported by Hjertereforeningen and Statens Lægemiddelstyrelse Forskningsråd. Technical assistance: Lis Anette Tøngård.

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U.E. 2. Incidence of Urinary Tract Infection at Autopsy Cases Treated for Bladder Cancer but without Cancer (urinary Renal Units (r.u.) 1st and without Dilated Pelvis and/or Ureters in Cases 1st Intestinal Conduit Diversion and 2nd Intestinal Diversion)

stage of infection	Cases with diversion		Cases without diversion	
	r.u. not dilated	r.u. dilated	r.u. not dilated	r.u. dilated
0	2	6	63rd	6
1	6	40th	94th	11
2	0	5	11	2
3	94th	104th	4th	74th
Total number	16	25	81	15

r = 1 r.u. with carcinoma
r = 0 r.u. with diabetes

r = 1 r.u. with papillary microcarcinoma (and above of phenacetine)
g = 1 r.u. with ureter transplanted in the bladder

7 of these were pyelonephritis with renal abscesses (Table 1) (Fig. 1).

Fifty cases without urinary diversion had no evidence of cancer at autopsy. 14 of them had urinary tract infection, 8 of which consisted of pyelonephritis with abscesses (Table 1). The difference in frequency of infection (renal abscesses and/or pus-filled pelvis/ureters and/or reddish mucosa in pelvis/ureters) between the cases with and without diversion is statistically significant (χ^2 -test, $p < 0.0005$). Conduit cases had renal abscesses more often than control cases (χ^2 -test, $p < 0.01$).

When we excluded cases with dilated upper urinary tracts, 14 of 16 renal units in cases with urinary diversion and 13 of 81 renal units without diversion had infection in the urinary tract at autopsy. The difference is statistically significant (χ^2 -test, $p < 0.0005$). The difference between the groups had the same level of significance if we also excluded cases with diabetes, urinary tract carcinoma, phenacetin abuse and ureteral transplantation in the bladder. In cases without diversion infection was found in 60 per cent of dilated renal units and in 16 per cent of non-dilated renal units (Table 2). The difference is statistically significant (χ^2 -test, $p < 0.0005$). No corresponding difference was found in cases with conduit diversions.

Conduit cases with cancer at autopsy had evidence of infection in 10 of 63 renal units (9 of 23 if dilated renal units are excluded). These figures imply a significantly lower incidence of infection in those conduit cases that had cancer at autopsy (cf. Table 2). This difference was not found in non-diverted cases.

TABLE 3 A. Incidence of Urinary Tract Infection at Autopsy in Conduit Cases 1st and 2nd Intestinal Diversion from Göteborg and from outside Göteborg are Presented Separately

B. Incidence of Cancer in Non-Autopsied Conduit Cases

A.	Autopsy cases			
	Cases from Göteborg n = 41		Cases from outside Göteborg n = 13	
	Infection stage			
	0	1	2	3
No cancer at autopsy	2	4	2	8
Cancer at autopsy	11	1	4	9
	12	5	6	17
	5	3	2	3
B.	Non-autopsied cases			
	Cases from Göteborg n = 18		Cases from outside Göteborg n = 22	
Metastases at X-ray	14		22	
Unknown cancer status	4		0	

n = patient dead in trauma with clinical diagnosis of renal infection

PATIENTS AND METHODS

Urinary diversions with an ileal conduit were performed in 197 patients at Sahlgrenska sjukhuset, Göteborg, 1963-1973. At the follow-up 94 were dead, 54 had autopsy records which permitted detailed analyses of the conditions in the urinary tract. They were all operated upon because of carcinoma of the urinary bladder. 33 of the 54 autopsies showed evidence of urinary bladder cancer locally or as metastases, whereas 21 did not. 18 of these 21 were men (mean age at operation 63 years, range 52-73) and 3 were women (mean age 58 years, range 29-76).

401 out of 954 patients treated for urinary bladder carcinoma but without urinary diversions at the hospital during the same period were dead at the follow up. Detailed autopsy records were available in 181 which are the control cases, 131 of them had cancer at autopsy and 50 had not, 35 of these 50 were men (mean age 65 years, range 55-85) and 15 were women (mean age 75, range 63-85).

Cystectomy had principally been performed in cases with medium-high differentiated cancer. However, cases older than 69 years were usually not cystectomized. Neither were cases with diabetes or serious cardiovascular diseases. All ureters were implanted in the conduits without reflux-preventing technique. Patients with anaplastic cancer had as a rule been treated with radiology and not cystectomized. Cases with highly differentiated tumors had been treated with transurethral resection of the tumor.

Evaluation of Autopsy Records

The autopsy records have been reevaluated by one pathologist. Macroscopical evidence of upper urinary tract infection has been divided into four stages.

Infection stage 0 No signs of infection i.e. normal mucosa, no purulent exudation, no renal abscess.

Infection stage 1 Reddened and marked mucosa usually with petechiae in the ureters and/or renal pelvis. No renal abscess.

Infection stage 2 Inflamed mucosa with definite purulent exudation in the ureters and/or renal pelvis. No renal abscess.

Infection stage 3 Pyelonephritis with macroscopical abscesses in the renal parenchyma.

Renal abscesses were suspected in some cases from stage 1 and 2 at autopsy and verified histologically but since histological examinations were not performed throughout these cases were retained in their respective groups. Chronic pyelonephritis or papillitis necroticans has not influenced the staging of the infection in this study for the same reason.

The ureters and pelvis were divided either as dilated or non-dilated. Only indisputable deviations from normal width were grouped as dilatation.

Case Record Observations

Urinary cultures. The last urinary culture, when taken within six months before the patient's death, has been

listed. $>10^5$ bacteria/ml was considered as significant bacteriuria.

Survival time after urinary diversion and the first treatment for bladder carcinoma in non-cases was compared. Cases older than 69 years at first treatment for bladder carcinoma were not patients of that age seldom were diverted. Cases surviving two months postoperatively were excluded in order to minimize the influence of operative mortality. The influence of age, sex and of radiological treatment the frequency of infection was studied.

Statistical methods. χ^2 -test, fourfold table test (Miettinen-Geigy) and Wilcoxon test were used for calculations.

RESULTS

Autopsy Findings

Twenty-one cases with a conduit had no evidence of cancer at autopsy. Eighteen of these macroscopical evidence of urinary tract infection.

TABLE 1 Incidence of Urinary Tract Infection in Autopsy Cases Treated for Bladder Cancer without Evidence of Cancer at Autopsy. 21 with Ileal Conduit Diversion and 50 without Diversion

Stage of infection	Cases with diversion	Cases without diversion
0	3	36
1	5	5
2	3	1
3	10	8
Number of cases	21	50



Fig. 1 Kidneys, ureters and ileal conduit from a 71 old man without cancer at autopsy. Dilatation of ureters and pelvis with purulent exudation. Pelvic abscesses. Necrotizing papillitis, often renal cortex abscesses. Infection stage 3.

without infection (6 of 53) but the difference is not statistically significant.

No difference was found in the frequency of urinary tract infection at autopsy between men and women or between right and left kidney. Neither could any difference be found between cases with and without radiological treatment or between different age classes.

Survival time The mean survival time for autopsied cases without cancer and with conduit urinary diversion was 20.4 months and for those without diversion 44.6 months. If cases with dilated upper urinary tracts were excluded the survival was 16.6 and 50 months respectively, a difference in survival time which is almost statistically significant according to the Wilcoxon test ($p < 0.10$). Conduit cases with cancer at autopsy had a postoperative survival of 13 months.

DISCUSSION

Uremia was found to be the cause of death in as many as 40 per cent of cases with uretero-sigmoidostomy who died without evidence of cancer (7). A high frequency of renal infection also following conduit urinary diversion is universally agreed upon but most studies published include malignant cases. However, Solowash *et al* reported 6 deaths out of 130 dead conduit cases with benign disease (13). 4 of these 6 patients died of sepsis. Ellis *et al* followed 41 patients with intestinal conduit for benign disease more than 5 years (4). 33 of them developed symptoms of pyelonephritis after the diversion operation. 3 of the 41 were reported dead. However, the cause of death in these cases was not stated.

Most diversion operations are carried out because of urinary bladder carcinoma. To assess the influence of the malignant disease when evaluating urinary tract infection in these patients, cases with and without cancer were studied separately. We found high frequency of urinary tract infection in conduit cases with cancer at autopsy and a significantly higher frequency in cases without cancer. This difference may be due to the fact that cancer cases had a shorter survival time and died without having time to develop serious urinary tract infection.

The fact that the control cases were older than uroctomized cases and that general diseases, such as diabetes, were more common in the control group than in conduit cases, should imply that control cases had urinary tract infections more often than conduit cases. The high incidence of bacteriuria pre-mortally in the controls may be explained by these factors. Thus, our finding of the

highest frequency of infection in conduit cases is not influenced by different age or diseases in the control group and seems to depend on the urinary diversion procedure. The finding of the same frequency of bacteriuria pre-mortally in autopsied conduit cases as in non-autopsied conduit cases argues against a selection of cases with urinary tract infection for autopsy. If there was a bias in selection of autopsy cases one could expect to find different frequencies of infection in groups of patients with different autopsy frequencies. However, our findings were not influenced by the autopsy frequency at different hospitals or different bacteriological findings in pre-mortally urinary cultures.

Both conduit cases with dilated and those with non-dilated upper urinary tract had high frequency of upper urinary tract infection at autopsy. The explanation may be ureteral reflux, which is an almost constant finding in conduit cases without reflux-preventing ureteral implantation, as reflux may initiate or maintain urinary infection. Other explanations may be a constant vesical stasis in the conduit or a bacterial invasion through the cutaneous stoma, as the natural defence mechanism of the urinary bladder and the urethra against infections is lost.

Dilated ureter and/or pelvis may result from stricture at the uretero-intestinal anastomosis or from ureteral orifice strictures at transurethral treatment of bladder cancer. Ureteral stricture is a well-known factor in the genesis of upper urinary tract infection. We therefore excluded every case with dilated pelvis and/or ureter and still found a significantly higher frequency of infection in diverted cases than in the others.

The higher frequency of infection in dilated renal units than in non-dilated ones in the control cases may be explained by ureteral stricture or by unilateral ureteral reflux, following transurethral resection. Alternatively the explanation may be that cases with infection develop wide upper urinary tracts as a result of established upper urinary tract infection (14).

It is difficult to evaluate the importance of the differences shown in frequency of upper urinary tract infection between conduit cases and controls, as 103 diverted patients and 553 controls were still alive. However, the high frequency of infection in the total material of diverted patients is indisputable. Excluding the 33 cases with cancer at autopsy and the 36 with roentgenologically verified cancer metastases from the 197 diverted patients, 128 remained. 18 (14%) of these 128 died without cancer but with infection in the upper urinary tract, 10 (8%) of them with renal abscesses, which is a remarkable finding. So is the fact that 45 per cent of

TABLE 4 Main Cause of Death as settled at Autopsy in 21 Cases with Intestinal Conduit Diversion and Evidence of Cancer at Autopsy

Cause of death	Survival time after diversion operation			
	< 1 month	1 month	≥ 2 months	0-3, years
Acute pyelonephritis			5	5
Pulmonary embolism	4			4
Intraabdominal abscesses			3 ^b	3
Peritonitis	3		1	2
Pulmonary oedema			2 ^c	1
Myocardial infarction	1	1		2
Pneumonia	1	1		2
Sepsis		1		1
No of cases	7	3	11	21

a, b, c = the 3 patients dead without evidence of upper urinary tract infection at autopsy (cf Table 1). They were all dead within one month after major surgery: a) cystectomy b) ileostomy c) on left ventricular ulcer of the ventricle.

In Table 3 the frequency of urinary tract infection in autopsy cases from the town of Göteborg and other areas are compared. The distribution of the different stages of infection was the same in both groups. The autopsy frequency was 69 per cent in Göteborg and 37 per cent in the other areas. 14 of the 18 non-autopsied cases from Göteborg and 11 of the 22 of the non-autopsied cases from the other areas had cancer metastases verified before death. Thus not more than 4 non-autopsied cases from Göteborg may have died without cancer and without infection. There was, however, clinical evidence of renal infection before death in two of these four cases as they had serumcreatinine values of 354 µmol/l and 972 µmol/l respectively and died with high fever.

Table 4 describes the causes of death as judged by the pathologist who performed the autopsy in the 21 cases operated upon with conduit diversion. 5 of the 11 (45%), who died more than 2 months

postoperatively had renal infection as the main diagnosis at autopsy.

Premortal urinary cultures. Autopsy cases with intestinal conduit had significant bacteriuria premortally in 89 per cent and non-autopsied cases with conduit in 81 per cent. The autopsy cases with intestinal conduit diversion had bacteriuria in 59 per cent (Table 5). The difference between non-diverted cases and diverted cases ($p < 0.005$) is significant. There is no difference in frequency between autopsied and non-autopsied conduit cases.

No difference in the frequency of bacteriuria could be shown between cases with and without evidence of urinary tract infection at autopsy.

Cases with infection at autopsy had had significant bacteriuria with growth of other bacteria than *E. coli* or coliforms (*Proteus*, *Klebsiella*, *Pseudomonas* or *S. albus*) more often (13 of 16) than cases

TABLE 5 Frequency of Significant Bacteriuria ($\geq 10^5$) at Last Premortal Urinary Culture in Cases with Intestinal Conduit Urinary Diversion with and without Autopsy and in Autopsied Cases without Diversion

	Cases with urinary diversion Autopsy n = 54	Cases without diversion Autopsy n = 181
	With urinary culture, n = 34	With urinary culture, n = 131
Significant bacteriuria	n = 30 (88%)	n = 81 (59%)
	Without autopsy n = 40	
	With urinary culture, n = 21	
Significant bacteriuria	n = 17 (81%)	

PLEURAL EFFUSION DISEASE IN RABBITS

Histopathological Observations

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Christensen, N., Fennessad, K. L. & Bruun, L. Pleural effusion disease in rabbits. Histopathological observations. *Acta path. microbiol. scand. Sect. A*, 86: 251-256 1978.

Pleural effusion disease (PED) is a generalized infection of laboratory rabbits caused by an unidentified agent, believed to be a virus. The histopathological response of 17 rabbits infected experimentally with this agent was studied. The light microscopical changes were minimal and the most consistent findings were alterations of the lymphoid tissue. Fatal infections were characterized by a uniform reduction of the splenic white pulp, focal degenerative changes of the thymus and lymph nodes and probably slight proliferative changes of the kidney glomeruli. In surviving animals there were transient myocardial and hepatic lesions and, after clinical recovery, proliferative changes in spleen, lymph nodes, metastatic lung lesions and probably kidney glomeruli. The results do not permit any conclusions to be drawn regarding the aetiology or the pathogenesis of PED infection.

Key words: Pleural effusion disease, rabbit, viral infection, histopathology.

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Received 11:78 Accepted 28:78

Pleural effusion disease (PED) is an infection of laboratory rabbits caused by a still-undefined agent. The disease was originally described in the late sixties as a mortality problem among rabbits used for the propagation of Nichols' pathogenic *Treponema pallidum* in Scandinavian laboratories employing the *T. pallidum* immobilization test, but the infection undoubtedly occurs under similar circumstances in other countries. Further investigations showed that the aetiology of the intercurrent disease was a virus-like agent, which apparently was being passed from rabbit to rabbit together with the Nichols strains (5, 6, 9).

Fennessad *et al.* (3) studied this passenger agent separated from the treponemes in serial rabbit passages and in a more detailed infection experiment. The disease, similar to the intercurrent disease, was characterized by a very short incubation period, fever, anorexia, leukocytosis, occasionally tachypnoea, and changes in the blood constituents. Most of the fatal cases occurred within 3-4

days, and the typical *post mortem* findings were pulmonary oedema and congestion, right-sided heart dilatation and large amounts of plasma-like fluid in the pleural cavities, hence the name pleural effusion disease. In fatal cases occurring after the first week of infection, peritoneal effusion was often also present.

So far the histopathological changes in PED have not been described. Jørgensen (7), who first reported on the intercurrent mortality among rabbits inoculated with the contaminated treponemes, mentions little or no changes among those surviving the double infection. In rabbits that died, hyaline thrombi were seen in the capillaries of the heart, surrounded by necrotic fibrils and eosinophilic cells, in addition to some degree of liver stasis and lower nephron nephrosis.

The present paper describes the histopathological changes in rabbits after experimental infection with the PED agent. The histopathology of the eye changes is not included in the present study but is reported elsewhere (4).

deaths more than 2 months after conduit diversion were due to acute pyelonephritis as determined by the pathologists at autopsy

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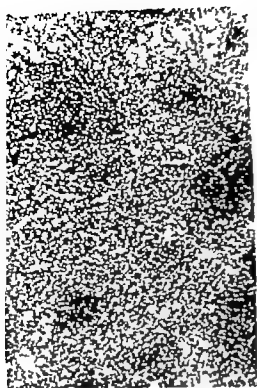
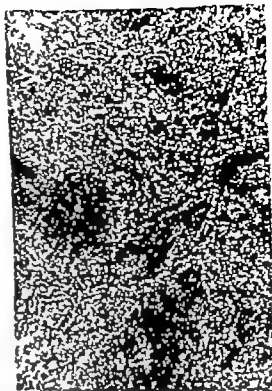


Fig 1 Spleen A Rabbit 1 p: day 3 Pronounced reduction in cellularity of the white pulp $\times 72$



B Rabbit 5 p: day 48 Proliferation of white pulp $\times 72$

normal when sacrificed on p: day 48. The five rabbits from the other infection experiment were sacrificed at various intervals after inoculation, either during their febrile or subfebrile stage of disease (nos. 13, 14 and 15) or shortly after having recovered from their clinical disease (nos. 16 and 17).

Table I summarizes the gross findings at death or sacrifice and the results of bacteriological examination. As shown in the table, oedematous and congested lungs, almost cell-free, protein-rich, pleural effusions, and right-sided heart dilatation were the most prominent gross findings in the seven fatal cases. Among the ten rabbits sacrificed, only one killed on p: day 5 showed pleural effusions. None of the other nine animals sacrificed on p: day 10 or later showed conspicuous gross changes.

Histopathology

Apart from evidence of circulatory disturbances in the fatal cases, the microscopical changes were confined to the myocardium, spleen, lymph nodes,



Fig. Thymus Rabbit 1 p: day 3 Numerous scattered foci of necrosis with phagocytosing macrophages $\times 72$

MATERIAL AND METHODS

Available for the study were 17 male albino rabbits (Ssc.CPH) aged 3 to 5 months, infected subcutaneously with similar doses of the PED agent. Animals nos. 1 to 12 represented one infection experiment, in which the rabbits were observed clinically until death or sacrifice on post inoculation (p.i.) day 48 (3). The remaining five animals (nos. 13 to 17) originated from another infection experiment, in which the rabbits were observed by ophthalmological examinations; these five rabbits had been sacrificed on p.i. days 5, 10, 10, 17 and 29 respectively (4).

Five rabbits (three males and two females), of similar age and from the same conventional stock as mentioned above, served as uninfected controls for the histopathological examinations.

The *post mortem* examinations were performed within 3 hours after death or sacrifice of the animals. All tissue specimens were fixed in 10 per cent neutral formalin. The paraffin embedded sections were cut at 6-7 microns, and the sections were routinely stained with Harris haematoxylin-eosin and van Gieson stain. Sections from

the central nervous system were also stained mainly by the Gallocyanin method. In addition, some sections, stained with P.A.S., Ziehl-Neelsen, van Gieson-blue and Grocott.

Sections from the following organs or tissues examined: cerebrum, cerebellum, medulla oblongata, thymus, lung, myocardium, liver, spleen, adrenal gland, mesenteric lymph nodes, duodenum, ileum and colon. In some animals the examination included the aorta, art. pulmonalis and pancreas. In description of the histopathological picture, particular attention was paid to the changes common to all examined at the same stage of disease.

RESULTS

All 17 rabbits developed a febrile disease on second day after inoculation, without any other symptoms having been observed. Seven of the rabbits from one infection experiment died on day 3 while the other five animals gradually recovered from their disease and appeared to

TABLE 1. Results of Examination of Rabbits Infected Experimentally with PED Agent

Group No	Rabbit No	Examined on day	Pleural fluid (ml)	Lung/body weight ratio ($\times 10^4$)	Other <i>post mortem</i> findings
I	8	3 (54)	74	96	Lungs: more or less oedematous and congested; slight local pneumonia in three animals
	9	3 (57)	7	57	Heart: right-sided dilatation in four animals
	10	3 (61)	6	117	Liver: mottled appearance in three animals; a small focal necrosis in one animal
	11	3 (67)	25	88	Spleen: slightly enlarged in six animals
	7	3 (63)	11	118	Mes. lymph nodes: slightly enlarged or haemorrhagic in five animals
	1	3 (66)	≥ 73	104	
	7	3 (67)	25	65	
<i>Bordetella bronchiseptica</i> from lungs of four animals; from one of these also anhaem. streptococci and coli from mes. lymph nodes.					
II	13	5	7	—	Lungs: normal or slightly oedematous and congested
	14, 15	10	0	—	Heart: right-sided dilatation in one animal
	16	17	11	—	Liver: slight chronic coccidiosis in two animals
	17	29	0	—	Spleen: slightly enlarged in two animals
	3, 4, 5, 6, 12	28	0	37-43	Mes. lymph nodes: slightly enlarged in one animal
					<i>Bordetella bronchiseptica</i> from lungs of one animal; no bacterial examination of Nos. 13 to 17

*Day refers to day after inoculation. Figures in parentheses indicate hours. †The rabbit died.
Group I: Pleural fluid protein ranged from 4.9 to 6.8 g/100 ml; total cell count in six of seven animals examined was less than 200 /cmm.

0 = less than 1 ml. — = not measured

sinus were normal as were the lungs and kidneys. However in the myocardium there were some small irregular areas of necrosis with a slight inflammatory response, and a focal interstitial infiltration of lymphocytes and polymorph leucocytes (Fig. 3). The blood vessels and the endocardial surface were normal and specific stainings failed to show micro-organisms. The three animals sacrificed on days 10, 10 and 17 also showed scattered, focal necrosis of liver cells, surrounded by mononuclear cells, whereas the portal tracts were unaffected (Fig. 4).

The remaining six rabbits in this group, sacrificed on days 29 or 42, had a definite hyperplasia of the white pulp in the spleen (Fig. 1 B), and there was an increased amount of interstitial follicular lymphoid tissue in the lungs. The follicular pattern in the lymph nodes was not exaggerated, and the thymus appeared to be normal. Cell counts of glomeruli suggested proliferation of endothelial and mesangial cells to a higher degree than seen in group 1 and in addition a few glomerular crescents were seen. There were no signs of inflammation, and the kidney tubules were unaffected.

DISCUSSION

The results of the present study show that the light microscopical alterations associated with PED infection of rabbits are minimal. Indeed some of the changes were demonstrable only after careful comparison with the uninfected control animals. This may be surprising, in view of the severe clinical picture and the gross findings in the fatal infections, but it is in accordance with the lack of gross changes in the animals sacrificed at intervals after the first week of disease.

The apparent discrepancy between the gross and the microscopical lung findings in the fatal cases raises a doubt as to the value of conventional histological preparations for the demonstration of lung oedema. The disappointing microscopical findings might suggest that a substantial part of the increase in lung weight was due to blood storage in the pulmonary vascular bed rather than to oedema of the lungs. Obviously this should be examined in other experiments, using a more suitable technique for the histological demonstration of lung oedema.

Bleeding circulatory disturbances, the fatal infections were characterized by a marked loss of cellularity of the white pulp of the spleen, focal degenerative changes of the thymus and lymph nodes and vasa proliferation of endothelial and mesangial cells of the kidney glomeruli.

The accumulation of a large amount of serum, pleural fluid with a protein content of 4.9 to 6.8 g

per 100 ml indicates an increased permeability of the pulmonary or perietal pleural capillaries or both (1). As reported elsewhere (3), an increased permeability of the so-called blood aqueous barrier was apparently present concomitantly in the eye. It is reasonable to assume that both these leakages were caused by the same mechanism.

Pleural effusions and a marked increase in the content of protein in the anterior eye chambers as seen in PED infection do not appear to have been reported in naturally-occurring or experimentally produced rabbit infections (10). However pleural effusions of comparable nature are well known in rats given alpha-naphthylthiourea (ANTU) (8, 2). In this poisoning, lung oedema is considered to be primary to the pleural effusions and to be due entirely to the formation of reversible gaps in the endothelium of the pulmonary capillaries and venules. Whether the same pathophysiological mechanism is operating in PED infection must be investigated in other experiments.

In surviving rabbits examined at different times after the onset of disease, the histopathological changes seemed to depend on the stage of infection. The animals examined during the clinical course of the disease showed a definite hypoplasia of the splenic white pulp as seen in the fatal cases, but furthermore there were focal necroses and inflammatory changes of the myocardium and from p.i. day 10 also necrosis of liver cells. The animals examined after clinical recovery from the disease showed proliferative changes in the spleen, lymph nodes, interstitial lung tissue and kidney glomeruli. The transient nature of some of the lesions also applies to the microscopically demonstrated viral reaction which regressed almost completely within four weeks (4). The alterations in the lymphoid tissue observed during the course of infection appeared to reflect different stages of a progressive reactive process, which is considered to be related to immunological phenomena.

The histopathological findings do not contradict the concept of a generalized viral infection affecting several organs.

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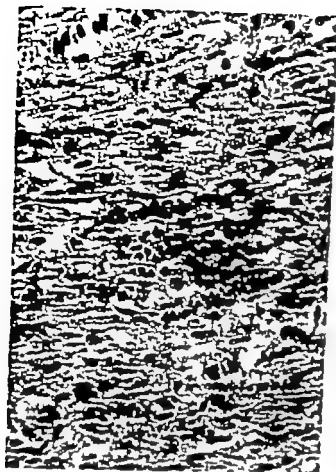


Fig 3 Myocardium Rabbit 15 p.i day 10 Focal interstitial inflammation with mononuclear cells and a few polymorph leucocytes $\times 180$

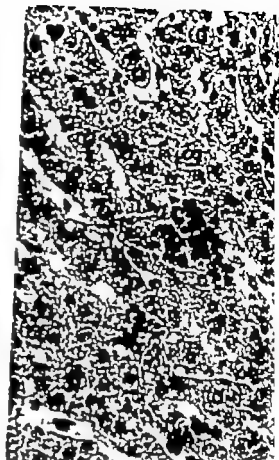


Fig 4 Liver Rabbit 14 p.i day 10 Focal liver necrosis with mononuclear cells $\times 180$

thymus, liver, lungs and kidneys. No changes were found in the central nervous system, adrenal glands, pancreas, large blood vessels and intestine. Specific granulomatous inflammation was not found and all attempts to demonstrate inclusion bodies, bacteria and fungi gave negative results.

In Table 1 the animals are divided into two groups according to spontaneous death or sacrifice. The histopathological changes will be described separately for each of these groups.

Group I These seven animals, which died 54 to 67 hours after inoculation, displayed a similar pattern of histological changes in the spleen, lymph nodes, thymus, kidneys and lungs.

There was an obvious reduction in the amount of white pulp in the spleen, as compared to the controls (Fig. 1 A). Small, scattered foci of degeneration or necrosis, often involving two or three cells, were found in the cortex and to a lesser extent in the medulla of both the thymus and lymph nodes (Fig. 2). These foci were either empty or filled with debris or macrophages. The lymph nodes also showed a moderate proliferation of histiocytes in the medullary sinuses, but the architecture of both thymus

and lymph nodes was well preserved. In the kidneys, cell counts of the glomeruli suggested a proliferation of endothelial and mesangial cells in all animals when compared with the controls. No inflammation was present and the tubules were normal.

In general, all organs from animals in this group showed pronounced congestion, particularly the lungs and liver. The lungs were slightly or moderately oedematous in scattered areas, but not to the degree anticipated from the gross examination. There were no signs of lung inflammation, and the amount of interstitial follicular lymphoid tissue was normal as compared with the control animals. **Group II** This group comprises 10 rabbits sacrificed on p.i. days 5 to 48. In these animals the alterations were confined to the spleen, lymph nodes, liver, kidneys, myocardium and lungs, but not all animals were equally affected.

Among the four rabbits sacrificed on days 5, 10, 10 and 17, the microscopical findings in the spleen were similar to those observed in group I, with a significant reduction of the white pulp. In contrast, other lymphoid tissues such as lymph nodes and

PLASMA MEMBRANE MOTILITY OF CULTURED HUMAN GLIA CELLS IN PHASE II AND III

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Blomquist, E., Arro, E., Brunk, U. & Westermark, B. Plasma membrane motility of cultured human glia cells in phase II and III. *Acta path. microbiol. immunol. Sect. A*, 86: 257-63 1978.

A human diploid glia line (U 787 CG) was cultured on hypotactic islands using a technique recently described. The surface morphology and motility patterns of the cells were studied using time-lapse cinematography and scanning electron microscopy. Proliferating cells showed locomotion and ruffling activity with intermittent evoked macroprotrusions. Non-dividing, i.e. cells which had not divided 10 days after seeding on the hypotactic islands, were immobile, larger than the dividers, rather flat with few microvilli, and showed only very occasional ruffles almost completely without evoked macroprotrusions. Our present findings show that phase III cells growing singly on hypotactic islands in serum-containing medium behave like phase II cells starved of growth factors or density growth inhibited. The results corroborate the theory that stationary cells have depressed ruffling activity and evoked macroprotrusions as compared with proliferating cells.

Key words: Cultured human glia cells, ageing *in vitro*, phase III phenomenon, plasma membrane motility, scanning electron microscopy, time-lapse filming.

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Received 22.78 Accepted 22.78

Normal diploid cells have a limited growth potential in culture. According to Hanflick (1965), the life span of serially propagated cells can be divided into three phases. Subsequent to explantation there is a period of slow initial multiplication (phase I) followed by rapid exponential growth (phase II), which eventually slows down before terminating in non-proliferation (phase III) and finally in cell death.

Cessation of growth *in vitro* has sometimes been compared with normal ageing *in vivo* and supposed to be an expression of senescence at the cellular level (Hanflick 1965; Craven & 1972; Hanflick 1966). The phase III phenomenon may also reflect an important growth regulation mechanism distinguishing normal from transformed cells. The latter can usually be cultivated indefinitely (Hanflick & Varmus 1961), and thus lack the inherent property of normal cells to sooner or later lose

their capacity to divide. The number of cell cycle passages, rather than the total time in culture (Daniel & Young 1971; Brunk *et al.* 1973), determines when the cells reach phase III. The maximal possible number of divisions of normal cells, however, seems to be influenced and modified by exogenous factors, since the time the cells remain in phase II has been shown to be dependent on the environmental conditions (Rian *et al.* 1975; Craven 1970; Rothermel & Green 1977).

Observations on fibroblasts (Merrill & Ross 1969; Craven & Sharp 1973), and human glia cells (Westermark 1978), have shown that any culture of these cells, regardless of passage number, is in fact a mixture of both cells with the capacity to divide (dividers or phase II cells) and cells incapable of further division (non-dividers or phase III cells) the proportion of the latter increasing with increasing passage level. This observation is in contrast to the older notion that the cultures are composed of cells

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PLASMA MEMBRANE MOTILITY OF CULTURED HUMAN GLIA CELLS IN PHASE II AND III

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Blomquist, E., Arro, E., Brunk, U. & Westermark, B. Plasma membrane motility of cultured human glia cells in phase II and III. *Acta path. microbiol. scand. Sect. A*, 86: 257-263 1978.

A human diploid glia line (U 787 CG) was cultured on heparin-coated islands using a microisland method recently described. The surface morphology and motility pattern of the cells were studied using time-lapse cinematography and scanning electron microscopy. Proliferating cells showed locomotion and ruffling activity with intermittent associated macrophagocytosis. Non-dividers, cells which had not divided 10 days after seeding on the heparin-coated islands, were markedly larger than the dividers, rather flat with few microvilli, and showed only very occasional ruffles almost completely without associated macrophagocytosis. Our present findings show that phase III cells growing singly on heparin-coated islands in serum-containing medium behave like phase II cells starved of growth factors or density growth inhibited. The results corroborate the theory that stationary cells have depressed ruffling activity and associated macrophagocytosis as compared with proliferating cells.

Key words: Cultured human glia cells, ageing *in vitro*, phase III phenomenon, plasma membrane motility, scanning electron microscopy, time-lapse filming.

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Received 22 II 78 Accepted 22 II 78

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Cessation of growth *in vitro* has sometimes been compared with normal ageing *in vivo*, and supposed to be an expression of senescence at the cellular level (*Hayflick* 1965, *Cristofalo* 1972, *Hayflick* 1966). The phase III phenomenon may also reflect an important growth regulation mechanism distinguishing normal from transformed cells. The latter can usually be cultivated indefinitely (*Hayflick* & *Moorhead* 1961), and thus lack the inherent property of normal cells to sooner or later lose

their capacity to divide. The number of cell cycle passages, rather than the total time in culture (*Daniel & Young* 1971, *Brink et al* 1973), determines when the cells reach phase III. The maximal possible number of divisions of normal cells, however, seems to be influenced and modified by exogenous factors, since the time the cells remain in phase II has been shown to be dependent on the environmental conditions (*Rosen et al* 1975, *Cristofalo* 1970, *Rhodes & Green* 1977).

Observations on fibroblasts (*Ver & Ross* 1969, *Cristofalo & Sharf* 1973), and human glia cells (*Westermark* 1978), have shown that any culture of these cells, regardless of passage number, is in fact a mixture of both cells with the capacity to divide (dividers or phase II cells) and cells incapable of further division (non-dividers or phase III cells), the proportion of the latter increasing with increasing passage level. This observation is in contrast to the older notion that the cultures are composed of cells

which are all capable of multiplying although with longer and longer cell cycle times as the cultures approach phase III.

We are currently studying the relationship between cell multiplication, membrane ruffling and ruffle associated pinocytosis. We have found these events to be linked in cultures of phase II human glia cells and also in glioma cells (Collins *et al* 1977). It would thus be of interest to explore whether differences exist in ruffling and associated macropinocytosis, between proliferating phase II cells and non-dividing phase III cells. Such an investigation must obviously be performed on sparse cultures in which neither proliferation nor membrane movement is density inhibited.

In order to be able to distinguish non-dividers from dividers under such circumstances, we utilized a modification (Hestermarck 1978, Harris 1973) of the method described by Carter (1967) by which cells can be grown on isolated 'haptotactic islands'. This method takes advantage of the fact that cells do not spread and attach to naked agarose (Hestermarck 1978) but do so readily if the agarose has been covered by palladium. When palladium is evaporated over an agarose surface, shadowed by an ordinary EM grid palladium islands, corresponding to the holes in the grid, are created. Seeded cells then settle on the palladium islands but not on the naked agarose strips. Since the cells cannot move across the borders of the islands, this method allows the observation over a period of several days of multiple cell clones. Growth kinetics and plasma membrane motility can thus be studied on individual selected cells or clones of cells for many days.

MATERIAL AND METHODS

Cell Lines and Standard Culture Conditions

All experiments were performed on diploid human glia cells of the line U-787 CG, passages 11 to 40. The cells were derived and kept in culture as described by Ponten and Macintyre (1968). The cells were grown on haptotactic islands (see below) in plastic 35 mm Petri dishes, some of which contained round 25 mm coverslips No. 0 (for time-lapse cinematography), or rectangular pieces of glass 12 x 6 x 1 mm (for scanning electron microscopy SEM).

Preparation of Haptotactic Islands

The haptotactic islands were prepared as described elsewhere (Hestermarck 1978). In the case of preparation of glasses for time-lapse cinematography or SEM the glasses were held at an angle of 45 degrees to the horizontal while the heated agarose was poured over them and immediately sucked off to ensure a thin layer of agarose.

For the preparation of the SEM-glasses it was found necessary to coat the glass surface with an evaporated

layer of palladium prior to the agarose coat. Otherwise the agarose layer detached from the glass during the dehydration in acetone.

Growth of Glial Cells on Haptotactic Islands

Stationary glial cultures were trypsinized and seeded in medium. The cell number was determined by electronic cell counter (Celloscope®) and 5×10^4 cells/ml were seeded into each Petri dish haptotactic islands. This inoculum, according to Poisson distribution, gave a high proportion of single islands (Hestermarck 1978). The medium was changed bi-weekly. Cell proliferation on the islands was determined every second day in an inverted phase contrast microscope (Daivert). A sufficient number of spots was analysed to ensure that at least 300 square initially single cells were identified.

Time lapse Cinemicrophotography

The 25 mm coverslips were mounted in a Dur Stotter chamber into which medium was injected. The medium of 37°C, gassed with air-CO₂ was used. Films were made on a Zeiss photomicroscope No. 1 using a phase planapochromatic x 40 oil immersion objective lens (NA 1.0) and a 100 mm rear camera was a Bolex Paillard 16 mm, controlled by Wild VBF B Control unit and a VBF-C timer. Kodak Ektar 16 Reversal 7176 films were used and camera developed. Occasionally the 35 mm camera of photomicroscope was used for taking micrographs of selected areas every 30 s for about 15 min. The equipment was placed in a walk-in incubator at 37°C.

Scanning Electron Microscopy

The cells were fixed in an aldehyde fixative, cross of 2% glutaraldehyde in 0.1 M cacodylate-HCl, 0.1 M sucrose (pH 7.2, total osmolality 510 mOsm, vehicle osmolality 300 mOsm) (Brunk & Eriksson 1977, Brunk *et al* 1975).

The fixation was initiated by slowly adding 2.5 ml of the fixative, prewarmed to 37°C, to the dish containing 2.5 ml of the medium while the cultures were still in the incubator. Care was taken not to move dishes, or change their pH or temperature, before fixation since ruffles have proven to be sensitive structures which easily withdraw when the cells are disturbed.

After five minutes, the medium-fixative mixture was sucked off, pure fixative at 37°C was added, and fixation continued for another 10 min at 37°C. The cultures were then removed from the incubator and fixation continued in a refrigerator for at least another 1 min. The cells were post fixed in 1% OsO₄ in 0.15 M Na-cacodylate-HCl and dehydrated in a graded series of acetone, starting with 70%. The cells were critical-point dried from CO₂ in a Polaroid II 1000 apparatus. The specimens were gold-sputtered in a Polaroid EQX sputter at about 0.1 torr, 17 kV and 20 mA, providing a 30 nm thick gold layer. The glasses were mounted on stubs with double-adhesive tape, and the edges sealed with silver conductive paint. The cultures were studied in a Jeol JSM I microscope at 10 kV or in a Jeol 100C

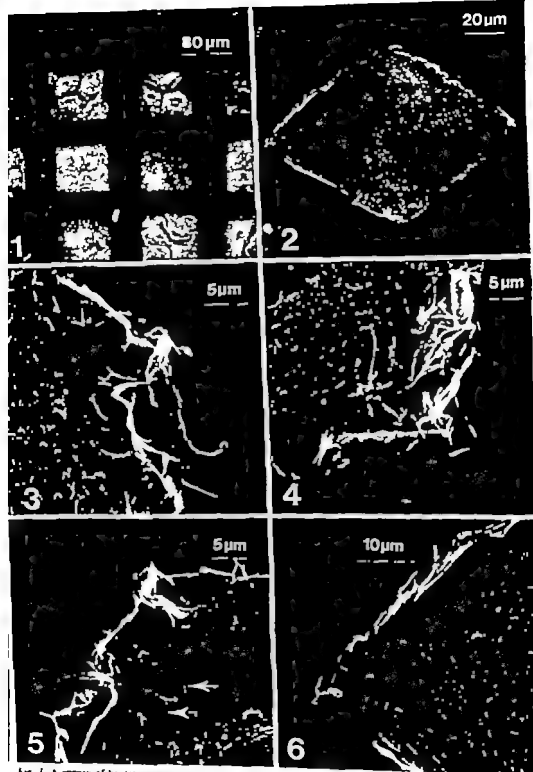


Fig. 1. A survey of hepatic squares containing dividers and non-dividers.

Fig. 2. Four dividers, almost completely occupying one square. The free cell margins show prominent ruffles.

Figs. 3-6. Details from divider cells showing complex ruffles with associated collapsed pericyclic vacuoles (arrows as on Fig. 5).

transmission electron microscope, equipped with a scanning attachment, at 40 kV. Micrographs were taken with the specimens tilted 45°.

RESULTS

Cell Growth Kinetics

Detailed results from the analysis of the growth of cell clones obtained from single cells in different passages are to be found elsewhere (Blomquist &

Westermark 1978). Most mitotic activity took place during the first few days after seeding. In the first passages (37-40) a small amount of mitosis can be found up to eight days after the initial seeding of cells on the haptotactic islands.

The fraction of non-dividers, i.e. cells remaining single on the square for ten days after seeding, increased almost exponentially with the passage number. In passage No. 11 18% of the population

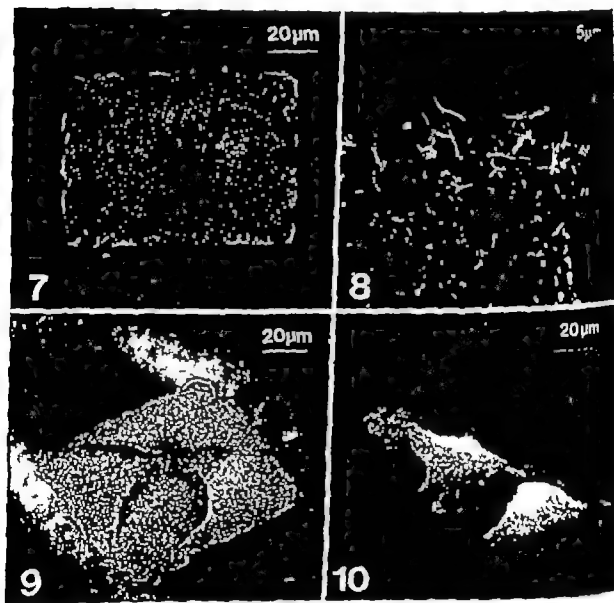


Fig. 7 A non-divider cell completely occupying one square. The cell is flat with a smooth upper surface.

Fig. 8 A corner detail of a non-divider showing membrane elevations at the peripheral border. No real ruffles are found.

Fig. 9 Dryer cells from passage 35 (close to phase III) showing low ruffling activity close to the appearance of non-dividers.

Fig. 10 Two dividers seen immediately after mitosis, showing intense ruffle activity at the outstretched lamellipodia. The rest of the square is occupied by a cell without ruffle activity.

found to be non-dividers. In passage No 40 the proportion had increased to 73 %.

The curve expressing the relationship between the percentage of nondividers and the passage number increased somewhat more steeply after passage No 40. In the following, cells which had undergone at least one division on the heptotactic islands were designated dividers (Fig 1).

Time-lapse Cinemicrophotography

Dividers from early passages showed rather intense ruffling activity with broad, fan-shaped ruffles built up of undulating lamellar structures. The ruffles were mainly oriented along the outer part of the squares, especially in the corners, since most squares were inhibited by two or more cells, so that contacts with neighbouring cells inhibited

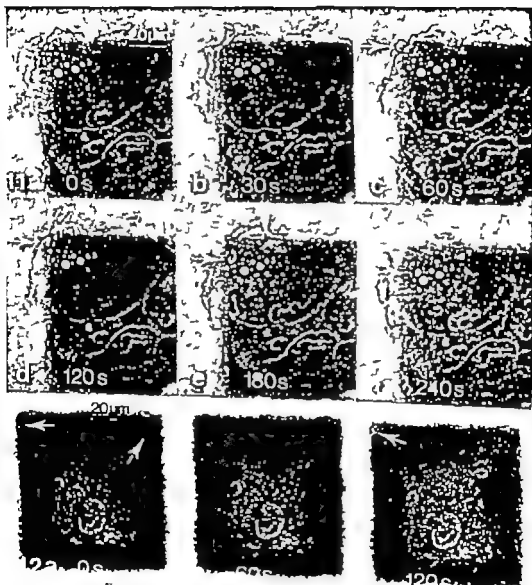


Fig 11 A sequence from a time-lapse film showing a cell from a logarithmically growing young culture. Note protuberant ruffling activity and associated formation of large pinocytotic vacuoles (arrows on Figs c-f).

Fig 12 A non-divider (fixed on a heptotactic square). The cell shows very limited membrane movements, no real ruffles are formed, no associated macropinocytosis.

ruffling of other parts of the cell periphery. The large ruffles were seen to give rise to clusters of macropinocytotic vacuoles forming during short intervals and moving towards the nucleus during absorption (Fig. 11 a-f).

Dividers from late passages also ruffled, but cells with very large and active ruffles were few. Most cells displayed a ruffling activity producing narrow and low ruffles. These cells showed macropinocytosis too, but it was obvious that the intervals between their formation were longer than in the case of dividers from early passages.

Non-dividers showed only a limited, undulating peripheral membrane activity. Thus motility was more an elevation of the peripheral parts of the cell than typical ruffles (Fig. 12 a-c). During a 24 hour long filming session, one or two somewhat larger ruffles could appear for a short time. Very occasionally such ruffles could be seen to result in a limited number (2-4) of pinocytotic vacuoles.

Scanning Electron Microscopy

Dividers from early passages in interphase were flattened and firmly attached to the substratum. The upper cell surface was rather smooth, except for a moderate number of delicate microvilli. Many cells showed broad, fan-shaped ruffles built up of wrinkled, erected lamellae, and were sometimes studied with tiny microvilli (Figs. 2, 9-10). Ruffles of different magnitude occurred, the most prominent ones consisting of two or three rows of lamellae (Figs. 3-6). Adjacent to such ruffles could be seen groups of caveolae which were interpreted as pinocytotic vacuoles which had collapsed during the dehydration and/or the critical-point procedure (Fig. 5).

Most dividers from late passages showed a type of ruffling activity which was intermediate between that of actively ruffling cells from early passages, and non-dividers. They had ruffles, but these were low and not prominent. There seemed to be a gradual shift of the type of ruffling cells capable of dividing in the cultures with increasing passage number from vigorously ruffling small cells to large and indolent ones showing little ruffling and low mitotic activity.

Non-dividers from both intermediate and late passages had the same appearance. They were much bigger than cells capable of dividing and most of them occupied the whole square. They also carried fewer microvilli on their upper surface, and generally gave a flat and quiescent impression. Ruffles were seen on only a few of these cells and were then of a rudimentary type. Elevation of the peripheral part of the plasma membrane, without formation of a typical ruffle, occurred more frequently (Figs. 7-8).

A population of cells seeded on small, identifiable, haplotypic islands isolated from each other but under identical culture conditions gave a system in which the growth of individual cells could be followed. The use of such a system made possible to combine kinetic and morphological studies on a comparatively large number of cells. In the present experiments, the cultures were followed for more than two weeks with a negligible cell loss and without signs of dislocation from one square to another. These results clearly demonstrate the occurrence of a fraction of non-dividers, present at all cell passage levels. The kinetics of the increase of dividers have been analysed in detail elsewhere (Blomquist & Westermarck 1978). Our findings are in agreement with those on WI 38 fibroblasts (Ross 1969; Cristofalo & Sharf 1973). It is obvious that any culture of human cells is probably of other diploid cells as well, is a true mixture of cells which are non-dividers (phase I cells) and dividers (phase II cells) but is however with widely varying mitotic rates remaining some being close to their entrance to phase III others having many generations left. This points to the danger of using mass cultures in various experiments in the belief that they cover a homogeneous population of cells with identical metabolism. Any study using cultures from well separated passages should of course be made since these will contain very different numbers of non-dividers.

Dividers from early passages showed ruffling activity and macropinocytosis. When dividers from early and late passages were compared, it was obvious that those from late passages showed less developed ruffles which only infrequently gave rise to clusters of macropinocytotic vacuoles. Furthermore, there seemed to be a gradual change of the median cell type in the cultures from cells with active and prominent ruffling during early passages, to cells which approached the appearance of non-dividers in late passages. This observation roughly paralleled the finding of prolonged doubling time required to obtain density-inhibited clonality of cells in the squares.

When compared with the dividers, the non-dividers appeared to be much less active in membrane movement. Thus, the non-dividers showed very little and the ruffles were not associated with macropinocytosis. They also had few microvilli, as has also been found on WI 38 cells in phase III (Bowman & Daniel 1975).

We have previously showed ruffles and

and macrophagocytosis to be related to cell multiplication and absent from quiescent glia cells blocked by serum starvation or high cell density (Collins *et al.* 1977, Brink *et al.* 1977). It was thus evident that the behaviour of nondividers with respect to ruffling activity was very similar to that of dividers blocked by serum-deprivation or density dependent growth inhibition.

Ruffling activity has, so far, been considered of importance for cell locomotion, pinocytosis and bulk uptake during phagocytosis (Harris 1969, Abercrombie *et al.* 1970, Lewis 1931, Gropp 1963). We would like to add a connection between ruffling and growth of anchorage-dependent cells, and feel that the findings of this study corroborate our previous hypothesis that ruffling and associated macropinocytosis are required for cell multiplication. Ruffling and associated pinocytosis may be an effective way of enhancing uptake of nutrients during the G₁ and S-phases of the cell cycle, or constitute a method for the turn-over of the plasma membrane which may need to be changed when the cells go through the cell cycle.

This work was supported by grants from the Swedish Medical Research Council and the Swedish Cancer Society.

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RENAL SEQUELAE TO NEPHROPATHIA EPIDEMICA

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Lähdevirta J, Collan Y, Jokinen E J & Hiltunen R. Renal sequelae to nephropathia epidemica. *Acta path. microbiol. scand. Sect. A* 86: 265-271, 1978.

Clinical data and renal biopsy findings were studied in 9 patients 4-5 years after the acute phase of Nephropathia epidemica. Laboratory data suggested slightly depressed tubular function, but glomerular function was normal. Light microscopy of renal biopsy specimens showed slight residual interstitial fibrosis and occasionally atrophic tubuli, tubular casts, increased number of hyaline glomeruli, and minor changes in other glomeruli. Two specimens showed fibrotic scars in the cortex. Electron microscopy revealed degenerative changes in the glomeruli, arterioles and tubules. The immunohistochemical study showed no fixed immunoglobulins in the kidney. The study shows that practically complete and lasting clinical recovery follows the acute phase of Nephropathia epidemica and that the disease does not leave diagnostic structural changes in the kidney.

Key words: Nephropathia epidemica, renal biopsy, electron microscopy, immunohistochemistry, renal sequelae.

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Received 9.11.77 Accepted 5.1.78

Nephropathia epidemica (NE) (Zetterhollen 1934, Al'lonson 1934 and 1945) is considered (Gajdinek 1953 and 1967, Lähdevirta 1971) a Scandinavian variant of the hemorrhagic fever with renal syndrome (HFRS, also called epidemic hemorrhagic fever or hemorrhagic nephroses-nephritis or Korean hemorrhagic fever), which occurs in Eastern Europe and the Far East. The etiology of this disease has not yet been finally clarified but probably is viral.

In Scandinavia the illness is milder and the mortality lower than in the Far East (0.2-0.5 per cent versus 5-10 per cent). The earlier clinical, histological and epidemiological study on NE in Finland (Lähdevirta 1971) included a mean 2-year follow-up of 20 patients, with light microscopy of 3 renal biopsies. In the present study of 9 patients the mean of the follow-up times is longer and 9 renal biopsies were studied by electron microscopy and immunohistochemistry.

MATERIAL AND METHODS

The patient series consisted of 9 males (age 22 to 45 years, mean 35.2 years) who had had the acute phase of Nephropathia epidemica 4-5 years before the present investigation. The disease had then been graded as mild in 2, moderate in 3 and severe in 4 cases (Table 1).

The combined test for endogenous creatinine clearance and excretion of phenolsulphonphthalein (PSP) was performed according to Rens et al. (1956). The renal concentration capacity was evaluated by means of the specific gravity of urine after 18 hours water fasting. Values over 1.023 were regarded as normal. Urinary pH values of 5.20 or below obtained spontaneously or after ammonium chloride load (0.1 g/kg of body weight) were considered to be evidence of a normal acid secretion capacity of the kidneys. Concentration and acidification capacities were tested twice and the better values were considered as representative (Table 1).

Paracentesis renal biopsies were performed with a modification of the Sierman needle. The specimens were divided in chilled Ringer's solution into three portions for light and electron microscopic and immuno-



Fig 1 Light micrographs of kidney biopsy specimens from patients who had had *Nephropathia epidemica* 4-5 years previously. Top: Interstitial fibrosis in the cortex. Trichrome stain, 220 \times . Middle: Interstitial fibrosis in the medulla but no inflammation. Trichrome stain, 275 \times . Bottom: Part of a scar in the kidney. van Gieson stain, 100 \times .

histochemical study. Samples for light microscopy were fixed in 4% formaldehyde (phosphate buffered) embedded in paraffin, sectioned at 4 μ m and stained with HE, van Gieson, methenamine silver PAS and Gomori's trichrome stains. Electron microscope samples were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer. The samples were embedded in Epon. Thin sections for light microscopy were cut and stained with methylene blue in 1% sodium borate. Ultrathin sections were stained with lead citrate and with 2% uranyl acetate in 50% ethanol. For immunohistochemical study, cryostat sections, 6 μ m thick, were stained by the direct technique with FITC-conjugated antibodies to IgA, IgG, IgM, C3 and fibrinogen. Monospecific antisera to alpha gamma and mu chains and fibrinogen were obtained commercially (Behringwerke AG). Otherwise the technique was identical with that described in an earlier paper (Runefors *et al.* 1971).

RESULTS

All patients were in good general condition and active in their former occupations (6 farmers, 2 drivers and 1 salesman). All but one patient had normal blood pressure. Glomerular function was good as judged by normal serum creatinine and endogenous creatinine clearance values. None of the patients showed proteinuria. Other data are presented in Table 1. They suggest in 5 cases slight residual tubular dysfunction.

Light microscopic examination of the renal biopsy specimen was performed in 7 cases (cases 2 and 6 excepted). All specimens contained renal cortex with 5–12 glomeruli per slide. There was no medulla in 3 specimens.

Hyalinized glomeruli were seen in all but one specimen (case 3) varying in number from 1 per 5

glomeruli to 5 per 12 glomeruli (the latter in case 5). Slight thickening of the basement membrane at the mesangial areas was seen in all cases. There was thickening of the capsular membrane in 1 in glomeruli in 5 cases. Slight periglomerular fibrosis was found in two of these patients. Atrophic tubules were found in fibrotic areas in 5 cases. Occasionally there were PAS-positive casts in the loops of Henle and brown pigment (lipofuscin) was seen in the epithelium. There was slight patchy interstitial fibrosis (Fig. 1) in the medulla in all 4 specimens containing medulla and in the cortex in 5 of specimens. Two patients (cases 5 and 9) had scars in the specimen (Fig. 1). Inflammation was scanty, a few lymphocytes were seen in fibrotic periglomerular areas.

PAS-positive thickenings were found in the arteriolar wall in 3 cases, and in one additional case there was a similar thickening in an interlobular artery.

In electron microscopic examination hyaline glomeruli showed light material with numerous membrane fragments accumulated inside the BV. The findings were similar to those described in obsolescent glomeruli (Nagle *et al.* 1969). In some glomeruli the BM was thickened at the mesangium and occasionally also in the walls of the peritubular capillaries. Occasional granular deposits inside the thickened BM (Fig. 2) contained membranous convoluted structures (Barley *et al.* 1974). Small structures could also be seen in thickened parts of the BM without deposits. Five of our patients had dark material in the mesangial areas (Fig. 3). The capsular membrane also showed dark deposits (Fig. 3). In the glomeruli especially in those in

TABLE 1 Follow up of 9 Patients after nephropathia epidemica

Case number	Age years	Time since acute phase of disease	Grade of clinical severity at acute phase	Blood pressure mmHg	ESR mm/h	Spontaneous rate of renal function after 18 hours water fasting	Lowest urinary pH	Secretion of PSP in 15 min (normal $\geq 25\%$)	Secretion of PSP in 60 min (normal $\geq 60\%$)
1	45	5 yr 2 mo	mild	115/60	5	10.4	5.05	5	7
2	42	—	—	170/80	11	10.7	5.9	45	84
3	22	4 yr 4 mo	moderate	135/80	3	10.27	4.93	18	77
4	35	—	—	140/75	2	10.1	5.10	9	71
5	37	4 yr 3 mo	—	150/105	1	10.1	4.13	30	77
6	30	5 yr 5 mo	severe	120/70	2	10.70	5	4	68
7	13	—	—	135/90	8	10.6	4.78	41	76
8	40	4 yr 5 mo	—	130/80	10	10.74	5.70	19	51
9	13	4 yr 4 mo	—	135/80	5	10.5	5.7	5	78

Clinical study of 9 male patients was performed 4 years 3 months to 5 years 5 months after acute phase of nephropathia epidemica. Borderline or pathological values are in italics. Serum creatinine and endogenous creatinine clearance values were normal and urinary protein was negative in all cases.

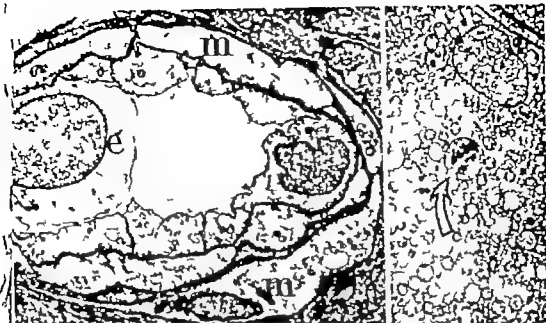


Fig. 4 A (left): Small interstitial arteriole of the corticomedullary area. Note the light and thick endothelial cells (e). The muscle cells (m) also are lighter than normal and show only a limited number of myofilaments (actin filaments); this feature suggests inactivity of the vessel. 4900 \times . B (right): A giant mitochondrion (arrow) in an epithelial cell of the proximal tubule. 3000 \times .



Fig. 5 An epithelial cell of the loop of Henle. Note the neuroendocrine cast (asterisk). The nucleus shows small vesicles of various sizes. 24000 \times . Inset: High magnification of small particles in cast inside a tubule of the distal part of the septum. 40000 \times .

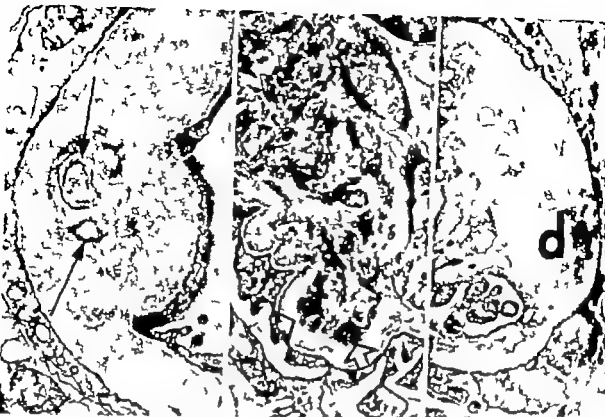


Fig. 2 A (left) Granular dark deposit inside the thickened peripheral glomerular basement membrane. Note the membranous convoluted structures (arrows). 14000 \times B (middle) Mesangial dark deposits (arrows) at the mesangial cell processes, often near the attachment bodies of the mesangial cells. 13000 \times C (right) Large dark granular deposit (d) in the mesangium 9800 \times



Fig. 3 Left. Capsular membrane with distinct dark granular deposits (asterisk). In the right lower corner there is a nucleus of an epithelial cell with a concentric inclusion (arrow). 7200 \times Right. Greatly thickened basement membrane of an arteriole. Note the muscle cells (m) with a faint basal lamina (arrows) in the wall of the vessel 10000 \times

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thickened capillary BM round extracellular particles (Bariety *et al* 1974) were seen between the epithelial cells and the BM. In two of our cases there were moon craters of the type described in other conditions (Osterby, Hansen & Olsen 1967; Sakaguchi *et al* 1965; Tallqvist *et al* 1976).

Medullary and corticomedullary arterioles were prominent and showed light and thick endothelial cells as well as light muscle cells with a limited number of actin filaments (Fig. 4). The interstitium showed fibrosis and occasional mast cells. In the cortex there was thickening and darkening of the arteriolar BM (Fig. 3).

In most of our cases occasional proximal tubule cells showed giant mitochondria (Suzuki *et al* 1975) (Fig. 4) or nuclear vacuoles. The casts in the distal tubules of the nephron contained membrane fragments embedded in light fibrillar or granular material. Many casts showed round or oval extracellular particles of varying diameter usually in the range of 50–100 nm (Fig. 5). In one sample small nuclear vesicles of varying sizes were also seen in an epithelial cell of Henle's loop (Fig. 5).

An immunohistochemical study was performed in all 9 cases. Neither immunoglobulin nor complement was observed in the glomeruli, tubuli or interstitium.

DISCUSSION

The prognosis after NE seems to be good. In our series all the patients were symptomless and in their former occupations 4–5 years after recovery from the acute phase. Only one patient had hypertension but its connection with NE was uncertain.

In 5 of 9 cases, however, there were slightly abnormal values in tubular function tests, though in 2 of these cases only borderline values in the concentration capacity. When added to the earlier follow up series of 20 patients (Lähdevirta 1971) this gives a material of 29 patients, 17 of whom showed borderline or abnormal values. 9 of these in two or more tests. This suggests that NE causes residual mild renal dysfunction in many patients. The tubular dysfunction is perhaps permanent but the glomerular dysfunction may heal slowly since the present series revealed no abnormal values in glomerular function, opposite to the earlier series (Lähdevirta 1971) with generally shorter follow-up times (mean $2\frac{1}{2}$ years). One explanation could be the scanty deposition of immune complexes in the glomeruli in the acute phase of NE (Ukkinen *et al* 1978) while the tubules and the interstitium are more severely affected.

The proportion of hyalinized glomeruli among all glomeruli in the biopsy specimens in the present

series was 19.6 per cent. In Jørgensen's data (1974) the corresponding proportion in biopsies from individuals without kidney disease was 5.5 per cent and in the same biopsies from individuals under 40 years 1.3 per cent only. We consider that the amount of interstitial scars in the present series is slightly increased. In electron microscopy we made several findings in glomeruli that were mentioned by Jørgensen (1966) in normal glomeruli. Membranous convoluted structures and small extracellular particles may be seen in later diseases and are probably due to degenerative phenomena, as suggested by Bariety *et al* (1974). Giant mitochondria occur in various disease states (Suzuki *et al* 1975). Dark deposits inside the basement membrane suggest immune complex deposits (Chung & Grishman 1975). In the present study immunohistochemistry was negative but the deposits are probably sequelae of immune complex deposition during the acute phase (Ukkinen *et al* 1978). Neither the mentioned changes nor the findings in the interstitial blood vessels and the intranuclear structures in tubule cells are considered to be specific for NE because they can also be found in other conditions.

Practically complete clinical recovery is the rule in NE. Mild renal tubular dysfunction, however, may remain in many cases, and renal biopsies reveal nonspecific mild changes, a proportion of which may be sequelae of NE and possibly connected with the tubular dysfunction as is known in other conditions (Berthne & Macken 1962). Clinical or immunohistochemical evidence of activity or progression of the changes has not been observed. Recovery is perhaps more complete in NE than in the more severe HFRS after which both depression of tubular function and increased morbidity in diseases of the urinary system have been reported (Rubin *et al* 1960; Sirovin & Lobastov 1976). Such increased morbidity after NE could not be documented in the present study or in the earlier investigation (Lähdevirta 1971).

The study was financially supported by The Serni Juselius Foundation and the Academy of Finland.

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RELIABILITY OF HISTO PATHOLOGICAL DIAGNOSIS OF SQUAMOUS EPITHELIAL CHANGES OF THE UTERINE CERVIX

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The reliability of histological diagnosis of squamous epithelial changes was tested by letting 13 pathologists read 100 consecutive cervical biopsies twice. Inter-observer and intra-observer agreement, variance, and deviation of diagnosis were determined. The diagnostic ability showed great individual variation and no significant correlation to experience in pathology. The diagnosis of invasive cancer had a high diagnostic specificity and the diagnostic sensitivity of the diagnosis of no significant epithelial changes was high too. The reliability of the diagnosis of dysplasia and carcinoma *in situ* proved satisfactory.

Key words: Histopathologic diagnosis, accuracy, quality control, reliability, observer error, cervix uteri, dysplasia, carcinoma *in situ*, carcinoma.

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Received 18:78. Accepted 18:78.

Rather few attempts at determining the accuracy of clinical judgments have been published. Most of these have revealed an astonishing lack of reliability (2, 5, 7, 9, 10, 13, 14, 24, 29). A histological diagnosis is nothing but a clinical judgment aided by histological technique and magnification in a microscope, like clinical judgments, most histological diagnoses do not rest upon exact measurements but upon subjective interpretation of often vaguely defined criteria, and many histological diagnoses are mainly the result of ill-defined pattern recognition. Therefore, it is only natural that investigations on the reliability of histological diagnoses, too, have revealed a considerable lack of consistency and

reproducibility (6, 23, 25, 26, 28). In view of the consequences of many histological diagnoses, further investigation of their accuracy was considered relevant.

Squamous epithelial changes in cervical biopsies were chosen as the object of the present study because of their clinical importance, the frequency of cervical biopsies in routine pathology and finally because the grading into dysplasia, carcinoma *in situ*, and invasive cancer render them ideal for quantitative analysis. ASHLEY¹ found that carcinoma *in situ* could be identified with certainty and with a low degree of observer error by any competent histopathologist (1). Other investigations on selected materials of cervical biopsies have

The average intra-observer agreement for the 13 participants was 89% (range 82-93%). If the diagnostic categories be compressed to 3 therapeutically relevant groups as shown in Fig. 3 the average agreement rises to 96% (range 92-98%).

- 1 2 No significant changes + slight dysplasia
- 3 4 Severe dysplasia + carcinoma *in situ*
- 5 Invasive carcinoma

Fig. 3 Therapeutically significant groups

Intra-Observer Agreement

Correlation diagrams with peer-diagnoses versus each reader's 1st diagnosis formed the basis for determinations of inter-observer agreements. As the 3 senior pathologists' diagnoses were used as a basis for the peer-diagnoses, only the remaining 10 participants' inter-observer agreement could be evaluated. The average inter-observer agreement proved to be only slightly smaller than the intra-observer agreement, viz. 87% (range 82-90%). If only the above-mentioned 3 clinically relevant groups of diagnoses be considered, the average agreement becomes 94% (range 92-96%).

The relatively high percentages of agreement in the present material were probably caused by its being a consecutive material including many normal biopsies. Therefore, the average agreement percentage was calculated after exclusion of all biopsies characterized as normal by peer. On this restricted material intra-observer and inter-observer agreements were as low as 68% (range 59-74%) and 51% (range 42-67%), respectively. If only the 3 therapeutically relevant groups of diagnoses be considered, these percentages are raised to 87% and 80% respectively.

The fact that most of the diagnostic uncertainty strongly concentrates on biopsies with epithelial changes made it interesting to test separately agreements on biopsies with a peer-diagnosis of invasive carcinoma. The average agreement with peer on the cancer-diagnosis was high, viz. 93% (range 87-100%).

Intra- as well as inter-observer agreement for the different readers showed great individual variation, but only a weak, statistically insignificant rise with increasing experience in pathology. The latter was measured as time spent in pathology departments. The 4 participating senior pathologists' intra-observer agreements, tested by double-blind readings averaged 90% (range 89-93%), as compared with 88% (range 82-92%) for the 9 younger doctors, this difference is statistically insignificant. The lack of correlation between diagnostic capability and experience was re-confirmed by comparing

each participant's performance on the first half of the biopsies with that on the latter half. The first 500 biopsies were read about 2 months before the last ones, and the agreements on examining the latter had not improved for any of the participants, not even for the one who had had only 1 month of experience in pathology when the investigation started.

For each reader agreement on double-blind reading (intra-observer agreement) was expected to be greater than his/her agreement with peer (inter-observer agreement) and that was confirmed by the present results. Intra-observer agreement and inter-observer agreement for the different pathologists, however showed mutual correlation, probably indicating that intra-observer uncertainty constitutes a substantial part of a reader's lack of agreement with peer.

Variance

Two pathologists obtaining the same percentage of agreement may theoretically differ in the spreading of the remaining diagnoses (17). As an expression of Dr Q's spread of his diagnoses around peer-diagnoses, a variance was therefore calculated according to the formula

$$S^2 = \frac{1}{n-1} \sum (x - \bar{x})^2$$

where n = number of biopsy diagnoses in the correlation diagram, $x = P - Q$, P = peer diagnosis, Q = dr Q's diagnosis, and

$$\bar{x} = \frac{\sum (P - Q)}{n}$$

The average inter-observer variance was 0.28 (range 0.20-0.49). The variance on double reading, calculated similarly for all 13 pathologists, averaged 0.23 (range 0.12-0.36). As with agreements there were great individual variations and no correlation to experience in pathology. The 4 participating senior pathologists had an average variance on double readings of 0.20 as compared with 0.25 for the younger pathologists. This difference is relatively small and it is not statistically significant. On the other hand, a significant correlation was found between intra-observer variance and inter-observer variance. Finally double readings as well as comparisons with peer showed correlation between agreement and variance.

Deviation

In order to analyse whether some pathologists systematically over-diagnosed or under-diagnosed the squamous epithelial changes, the average

shown uncertainty in the interpretation of squamous epithelial changes (3 4 8 12 14 15 16 20 22 27) However the magnitude of the observer error in ordinary routine materials remains unknown Therefore, an analysis of a large, consecutive material was attempted It was planned to determine intra-observer consistency by double blind readings and inter-observer reproducibility and diagnostic probabilities by comparisons with reference diagnoses The material did not allow an analysis of two major problems in histopathological work viz. sampling error and clinical correlation

On the other hand, the participation of 13 pathologists reading the same biopsies made it possible to relate the readers' diagnostic accuracy to their experience in pathology

MATERIAL AND METHODS

1001 consecutive cervical biopsies (March through December 1972) from Odense University Hospital were randomized and read blindly twice by each of 13 staff members at the pathology institute Four of the participants were senior staff members, the remaining participants training in pathology varied from 1 month to 6 years At each reading the participants had to place the biopsies in one of the categories of the Vienna classification (Fig. 1) Group 0 included biopsies without squamous epithelium group 1 included metaplasias reserve cell hyperplasia etc group 5 included adenocarcinomas

Evaluation of inter-observer accuracy necessitated a reference diagnosis for each biopsy For the purpose of the present investigation a clinical follow up of the patients could not be used Instead a peer-diagnosis on each biopsy was established in the following way in 862 cases the 6 diagnoses made independently by 3 of the participating senior pathologists allowed a decision of peer-diagnosis on the basis of complete or major agreement the latter requiring a minimum of 4/6 complete agreement and a maximum of 1 degree of deviation in diagnosis In the remaining 139 cases the 3

0 Unsatisfactory for diagnosis	82
1 No significant epithelial changes	716
2 Slight dysplasia	40
3 Severe dysplasia	68
4 Carcinoma in situ	40
5 Invasive carcinoma	55

1001

Fig. 1 Diagnostic categories according to Vienna classification. 0 includes biopsies without squamous epithelium, e.g. simple erosions 1 includes metaplasias, reserve cell hyperplasia etc., 5 includes adenocarcinomas The figures at right give the distribution of peer diagnoses, used as reference for determination of inter-observer variation and diagnostic probabilities

senior pathologists blind readings revealed no disagreement and a peer-diagnosis was then made by renewed reading by the 3 senior pathologists in concert The distribution of the peer-diagnoses is shown in Fig. 1 and illustrates the composition of the material

In accordance with the recommendations by Ben *et al.* (17) the following estimates of accuracy were calculated: Agreement, variance, and deviation

RESULTS

Intra-Observer Agreement

For each of the 13 pathologists the two diagnoses on each biopsy were transferred to correlation diagrams with the omission of all biopsies which either 1st or 2nd reading had been interpreted as unfit for diagnosis. Thus had the consequence that the 13 correlation diagrams contained different numbers of biopsies. An example of one of the correlation diagrams is shown in Fig. 2 For each reader the percentage of total agreement was calculated from the diagrams after adding the figures in the hatched diagonal.

	Dr Q's 1st Diagnosis					
	1	2	3	4	5	Total
1 No changes	632	40	11	0	1	684
2 Slight dysplasia	21	16	10	0	0	47
3 Severe dysplasia	7	5	37	4	0	53
4 Carcinoma in situ	2	0	11	30	2	45
5 Carcinoma	1	0	0	3	48	52
Total	663	61	69	37	51	881

Fig. 2 Example of correlation diagram with one participant's double-blind readings of 1001 consecutive cervical biopsies. Biopsies that on 1st and/or 2nd reading were designated unfit for diagnosis have been omitted

Figures in the hatched diagonal are the numbers of biopsies that got the same diagnosis on 1st and 2nd reading by Dr Q

we may now calculate the desired probability for each of the diagnoses dysplasia, carcinoma *in situ*, etc. (Fig. 5) If we compress the diagnostic categories to the 3 therapeutically relevant ones we get directly the most interesting diagnostic probabilities (Fig. 6) viz. the diagnostic sensitivity (predictive value of a diagnosis of slight dysplasia or no significant changes), 96% and the diagnostic specificity (predictive value of a diagnosis of invasive carcinoma), 94%.

DISCUSSION

The diagnostic probabilities found in the present investigation are not directly applicable to routine performance in pathology institutes. In daily routine the reliability of diagnoses will probably be higher for the following reasons: biopsies will be treated more carefully than in a boring examination – twice – of 1001 biopsies such as the present one – most slides – and especially those giving rise to problems – will be discussed between colleagues, and finally errors caused by demanding strict diagnostic categories and by coding for electronic data processing will not occur. Those cases of differing opinion as to the diagnosis of invasive cancer that did occur among senior pathologists in the present study had all been diagnosed correctly in the previous routine work on the same biopsies. Thus it can be assumed that the diagnostic sensitivity of a diagnosis of no significant epithelial changes as well as the diagnostic specificity of a diagnosis of invasive cancer in routine pathology is close to 100 per cent. The diagnostic probabilities of dysplasia and carcinoma *in situ*, however, are highly unsatisfactory. Reasons for this may be sought in the training of pathologists and in the nature of the diagnostic categories we are using. An exceptionally low standard of the pathologists participating in the present study is a tempting but probably not a sufficient explanation, whereas the great individual variation in diagnostic performance should be emphasized. The almost complete lack of correlation between diagnostic ability and time spent in pathology indicates that simple «learning by doing» – which is the major principle of education of Danish pathologists – is insufficient. Individual data like those obtained in the present investigation can be used as a basis for special training. Some will undoubtedly use the results of the present study as an argument for the introduction of a specialty board examination in Denmark with appropriate tests of diagnostic ability. Quite another and maybe the most important reason for the poor results on dysplasias and carcinoma *in situ* might be that these diagnostic categories are too vaguely defined. It is well known that the Vienna

definition of carcinoma *in situ* is not universally agreed upon (11–21), which will inevitably increase uncertainty. Few pathologists consider the names of the epithelial changes on cervix as expressions of true biological entities, most probably they suspect that dysplasias and carcinoma *in situ* merely represent an artificial systematizing of something that nature created as a continuum. If future efforts in the training of pathologists and/or establishing more strict diagnostic criteria fail to increase the accuracy of the histo-pathological diagnosis of precancerous lesions of cervix uteri, a reduction of the present diagnostic categories should be seriously considered.

Before new pathologic entities are introduced in routine pathology they should ideally be tested by double readings for their being consistently diagnosed, different pathologists – not only «experts» – should have proven able to reproduce the diagnoses, and finally the clinical relevance of new diagnostic entities should be secured. The latter demand is most often fulfilled even in preliminary reports in the literature, whereas the former two are very often lacking, even before therapeutical consequences are drawn from their routine use on patients. In the field of exfoliative cytology a great number of publications have dealt with diagnostic probabilities, usually with histological diagnoses as reference diagnoses, however assessments of observer error in cyto-pathology are still scarce (19).

One may ask why tests of the reliability of macroscopical diagnoses are so scarce. The reason is probably a historical one. It is likely that the first attempts at improving tumour diagnosis by histological technique and microscopy in the middle of the 19th century showed such an obvious superiority over clinical judgements that the magic term «histologically verified» was accepted without further proof. This state of affairs has remained practically unchanged ever since. The present investigation justifies the magic of this term as far as the reproducibility of the histological diagnosis of invasive carcinoma of the cervix is concerned. However the introduction of new techniques producing very small amounts of cells or tissue and the increasing interest in detecting early cancer *in situ* forms, and in other fields of pathology often very subtle changes, call for a systematic effort for testing the diagnostic reliability of cyto- and histopathology.

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deviation of each pathologist's diagnoses from peer diagnoses was determined in the following way. For each biopsy the difference: Peer-diagnosis minus dr Q-diagnosis (positive or negative) was determined, all differences were added, and dr Q's average deviation calculated

$$\left(\sum \frac{P - Q}{n} \right)$$

Two of the 10 pathologists had a negative average deviation, i.e. they had overestimated the squamous epithelial changes compared to peer. The remaining 8 pathologists had a positive average deviation, i.e. they had underestimated compared to peer. The average deviations were all smaller than 0.1, however they should not be considered insignificant as five of the pathologists had an average deviation from peer-diagnoses greater than 3 times the standard error calculated on the basis of the inter-observer variances for the said pathologists. Neither the size nor the direction of the deviation correlated to experience in pathology.

Diagnostic Probabilities

The gynecologist – and the patient – will want to know how much they can rely upon the pathologist's diagnosis on a cervical biopsy. Their interest

	Peer diagnosis					Total
	1	2	3	4	5	
1 No changes	6576	205	128	32	17	6953
2 Slight dysplasia	262	112	139	23	2	538
3 Severe dysplasia	50	62	299	112	10	533
4 Carcinoma in situ	11	3	97	216	16	343
5 Carcinoma	11	2	5	12	495	525
Total	6910	384	668	395	535	8892

Fig 4 Correlation diagram with all the diagnoses made on 1st reading by 10 ordinary pathologists versus peer diagnoses. Pairs of diagnoses including the diagnosis of unsatisfactory material have been omitted.

		Diagnostic Probabilities				
		1	2	3	4	5
Ordinary Diagnosis	1 No changes	94%	3%	2%	<1%	<1%
	2 Slight dysplasia	49%	21%	16%	4%	<1%
	3 Severe dysplasia	9%	12%	56%	21%	2%
	4 Carcinoma in situ	3%	1%	28%	63%	5%
	5 Carcinoma	2%	<1%	1%	2%	94%

Fig 5 Diagnostic probabilities. To be read as follows: with uppermost row as an example: If ordinary diagnosis is 1 (no changes), the probability of peer-diagnosis being the same is 94%, the probability of a peer-diagnosis being slight and severe dysplasia is 3% and 2%, respectively, the probabilities of peer-diagnoses being carcinoma in situ or cancer are both less than 1%.

may be phrased as the question: What is the probability of an ordinary diagnosis being true? We have chosen the peer-diagnoses as the best approximation to truth, and we can denote the remaining 10 pathologists' diagnoses as ordinary diagnoses. On the basis of a correlation diagram containing all ordinary diagnoses versus peer-diagnoses (Fig 4)

		Diagnostic Probabilities		
		1 + 2	3 + 4	5
Ordinary Diagnosis	1 + 2 No changes and slight dysplasia	96%	4%	<1%
	3 + 4 Severe dysplasia and carcinoma in situ	14%	83%	3%
	5 Carcinoma	2%	3%	94%

Fig 6 Diagnostic probabilities with diagnostic categories compressed to 3 therapeutically significant groups. Diagnostic sensitivity (predictive value of the diagnosis: Slight dysplasia or less) 96%. Diagnostic specificity (predictive value of the diagnosis: Invasive cancer) 94%.

HEART AUTOPSY FINDINGS IN CASES OF SUDDEN DEATH

The Distributions of Infarctions, Coronary Stenosis and Thrombi

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Østergaard, K. Heart-autopsy findings in cases of sudden death. The distributions of infarctions, coronary stenosis and thrombi. Acta path. microbiol. scand. Sect. A, 86: 279-284, 1978.

The pathological heart changes observed have been recorded at necropsy of 55 male and 31 female patients who died during transport to the hospital or in the casualty ward. The extension of acute myocardial infarction (AMI) was determined by a staining method using nitro-BT, and the infarctions were classified as transmural, combined or subendocardial. A total of 45 AMI's were found, and this is a lower frequency than normally found in sudden unexpected deaths when only death certificates are studied. The most frequent infarction type was the subendocardial infarction, which was present in 60% of the AMI-cases. Coronary thrombi were seen in only 24% of the AMI-cases, most frequently together with transmural infarctions.

Key words: Early infarction, Nitro-BT, coronary thrombi, sudden death.

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Received 12.1.77 Accepted 28.1.78

The purpose of the investigation, of which this study is a part, is to determine the possible causes of death, the spectrum of pathological changes and the distribution of trace elements in the organism in a group of patients who had died suddenly. In this study the pathological changes found in the hearts are reported. Special attention has been paid to early acute myocardial infarction (AMI) and coronary stenosis.

MATERIAL AND METHODS

The material comprised persons who died suddenly in the casualty ward or during transport to the hospital (Sundby, Copenhagen) in the period 1.3.1974 to 30.10.1974. A total of 152 «sudden» deaths occurred during this period, but autopsy was refused for religious reasons in 28 cases, 31 cases were transferred to legal autopsy and a further 7 cases were excluded as they were foreigners, leaving 86 cases for study.

The distribution according to age and sex is shown in Table 1. The 55 males had a mean age of 68.3 years (S.E. = 1.42 years) and the mean age of the 31 females was 70.2 years (S.E. = 2.21 years). The age differences is not statistically significant.

All subjects were autopsied by the author and the examination included brain-autopsy and histological examination of organs and the various types of tissue. The heart weight was determined, the coronary arteries were cut lengthwise and examined, and the degree of stenosis was classified according to the WHO-standard (1958) as moderate stenosis (more than 50% of the lumen preserved) and severe stenosis (less than 50% of the lumen preserved). The localization of thrombi if any was recorded, and cross-section of thrombus and blood-vessel was made for histological examination. The heart was then cut transversely by hand into approx. 10 mm thick slices from the apex to the apex of the papillary muscles. Each slice was examined, and the extent and localization of both recent and old infarctions were recorded. On one slice from the central third of the heart the enzyme-histochemical test using nitroblue tetrazolium (nitro-BT) according to *Anchels & Shorika* (1963)

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Old infarcted areas were located macroscopically and confirmed microscopically. They were found much more frequently in the AMI group (71%) than in the non-AMI group (29%); this difference is significant ($P < 0.01$). Old infarcted areas were found most frequently together with subendocardial infarctions (78%), a little less frequently (69%) together with combined infarctions, and least frequently combined with transmural infarctions (0%) but the differences are not significant. Of the 3 old infarctions observed, 32 were classified as subendocardial whereas 16 were either transmural or combined infarctions.

Subrecent infarctions (more than 2 weeks and less than 1 year old, according to Mallory *et al.* 1939) were seen at the histological examination in

14 of the 45 AMI cases. They were seen significantly ($P < 0.01$) more frequently combined with transmural infarctions than combined with subendocardial infarctions.

Infarction of one papillary muscle was seen in 13 cases (in $p = 0.69$ not significant) most frequently in the group with transmural infarctions, and not observed together with combined infarctions. Of the infarctions, 4 were of the anterior papillary muscle, whereas 9 were of the posterior papillary muscle.

Infarction of two papillary muscles, on the other hand, was seen most frequently together with combined infarctions (92%), and only in one of the 5 cases with transmural infarctions. Infarction of two papillary muscles was seen in 23 cases (62% in $p = 0.82$, not significant).

TABLE 2 *Salient Autopsy Findings in Relation to Infarction Types*

	Types of recent infarctions				No infarction
	Transmural	Combined	Subendocardial	Total	
Males	3	9	21	33	22
Females	2	4	6	12	19
Total	5	13	27	45	41
Thrombi					
fresh	4	4	3	11	—
old	1	3	9	13	4
Degrees of arterial stenoses					
more than 1 cor. art.	2	2	9	13	11
50% narrowing 2 cor. art.	1	4	8	13	6
seen in 3 cor. art.	2	5	9	16	6
Old infarctions	2	10	21	33	15
Subrecent infarctions	4	5	5	14	—
Infarction of					
1 papillary muscle	3	0	10	13	—
2 papillary muscles	1	12	15	28	—
Infarction of the right ventricle	2	4	2	8	—
Infarction of the septum	1	6	6	13	—
Heart weight					
more than 400 g	4	10	22	36	28
more than 500 g		6	12	20	10

TABLE 1 *Distribution According to Age and Sex*

Age in years	Males	Females	Total
35-39	0	1	1
40-44	0	0	0
45-49	1	1	2
50-54	2	2	4
55-59	11	2	13
60-64	7	4	11
65-69	12	2	14
70-74	7	4	11
75-79	5	9	14
80-84	7	3	10
85-89	2	3	5
90-94	0	0	0
95-100	1	0	1
Total	55	31	86

was applied, and the extent and localization of the fresh infarctions were determined. Furthermore, the thicknesses of the left and the right ventricular walls were measured.

From each heart the following samples were taken for histological examination: one transmural from the anterior wall and one from the posterior wall, both including the papillary muscles; one wedge-shaped sample from the septal wall was taken with known orientation. All infarcted areas were sampled.

The statistical analysis was performed using a simple *t* test to compare means, and either chi square tests (samples of more than 10) or fourfold table tests (Geigy 1970) (small samples) to compare proportions. Throughout the text the proportion of males (*m.p.*) has been calculated as a figure between 0 and 1.0 (e.g. 70% males equals a *m.p.* = 0.7).

RESULTS

Acute myocardial infarction was observed directly in 34 cases, and in another 10 cases the nitro-BT test disclosed infarction. In all the 34 cases with visible infarction this also showed up in the nitro-BT test, although the infarcted areas in most cases appeared to be bigger using the test. In none of the 10 cases where infarction could only be seen using the enzyme test did the histological examination contradict the enzyme test. In one case the histological examination showed infarction in the subendocardial part of the septum although this was not visible either with or without the enzyme test. This case has been regarded as a subendocardial infarction.

The male proportion (*m.p.*) of the 45 cases with AMI (0.73) is significantly ($P < 0.01$) different from

m.p. = 0.5 and also from the *m.p.* = 0.54 of the 41 cases without AMI. The mean age of the men with AMI (67.0 years) is lower than that of the females (74.1 years): this difference is not significant. The differences in mean ages between the group with AMI and the group without are not significant either in the male or in the female group.

The infarctions have been classified into three groups: transmural infarctions, subendocardial infarctions and combined infarctions, according to the following definitions: 1) Transmural infarction: necrosis of the left ventricular wall, including the septum at some point extending throughout the ventricular wall. 2) Subendocardial infarction: necrosis of the left ventricular wall not extending beyond the inner half. 3) Combined infarction: concomitant transmural and subendocardial necrosis in different parts of the left ventricle.

The salient findings are summarized in Table 2. The dominating infarction type was the subendocardial which was found in 27 (*m.p.* = 0.78) cases, or 60% of the 45 AMI cases. The next largest group was the combined infarctions, which were found in 13 cases (29% *m.p.* = 0.77) whereas transmural infarctions were only seen in 5 cases (11% *m.p.* = 0.6). The differences in male proportions are not significant.

Fresh thrombi as judged by macroscopical observation and confirmed microscopically were found in 24% of the AMI cases. The male proportion was 0.87 which is not significantly different from the *m.p.* of the remaining AMI. Fresh thrombi were seen most frequently combined with transmural infarctions, less frequently together with combined infarctions and least frequently combined with subendocardial infarctions. The differences in relative frequencies are significant at the 0.01 level.

Old thrombi were found in 29% of the AMI cases but only in 10% of the subjects without AMI. The difference in relative frequency is significant at the 0.05 level. Old thrombi were found with increasing frequency going from transmural through combined to subendocardial infarctions, but the differences are not significant.

The degree of stenosis has been assessed in determining whether one, two or three of the coronary arteries showed more than 50% narrowing. The degree of stenosis showed no significant dependence on infarction type. A comparison of the AMI group with the non-AMI group shows that AMI is more frequent the higher the degree of stenosis. The proportion of AMI cases with three coronary arteries more than 50% narrowed differs significantly ($P < 0.05$) from the proportion of non-AMI cases with this degree of stenosis.

he patients in our study all died suddenly and is also the case in the material obtained at ish medico-legal autopsies studied by Andersen (1976) Andersen & Hansen (1972) scraped hearts from patients who died in the real ward of Sundby Hospital, whereas the ones in Erhardt's (1974) material all died in a coronary care unit. This difference in the materials is explain the differences in the frequency of the infarction types if, as proposed by Spain (1972) and by & Holbørg (1972), all infarctions start at a subendocardial site and become transmural when a embolus forms.

Coronary artery thrombosis. The frequent finding of coronary thrombosis in patients with transmural infarctions as opposed to subendocardial infarctions is well recognised (e.g. Roberts & Baga 1972). As transmural infarctions are rare in the present material it is not surprising that coronary artery thrombosis is also relatively rare. Recent and old thrombi are found in 43% of the AMI cases, and this is significantly ($P < 0.05$) less frequent than in a study by Erhardt's (1974) (73%). This finding is in accordance with those of Heckel *et al.* (1969), who found that patients treated in a coronary care unit had coronary thrombi more often than patients in the general hospital wards. It also supports the theory of Ekel & Holbørg (1972) referred to above.

Degree of stenosis. It is generally accepted that coronary stenosis is a contributing factor in the aetiology of myocardial infarction, and our observations agree with this. The relation between the degree of stenosis and the types of infarctions is not been extensively studied elsewhere. Our results support the findings of Roberts & Baga (1972) that no such relationship exists. The finding that myocardial fibrosis is more pronounced the higher the degree of stenosis is as would be expected.

Old infarctions. The old infarctions were predominantly subendocardial (66%) and this corresponds to the findings of Erhardt (1974). He did, however, also find old infarctions more often when the fresh infarction is subendocardial. This is not supported by the observations reported here that old infarctions are seen with equal frequency in both the combined and subendocardial groups, and not significantly less frequently in the transmural group.

Subacute infarctions. Our observation that subacute infarctions are found more frequently combined with transmural infarctions than with subendocardial infarctions also lends support to the theory that transmural infarction is a secondary phenomenon.

Papillary muscles. Fresh infarctions of one or

both of the papillary muscles were found in 91% of the cases with AMI. This is a high frequency compared to what has been found in previous studies. Erhardt (1974) found fresh papillary muscle infarctions in 68% of 84 cases, and this is a higher percentage than found by e.g. Brand *et al.* (1969) who found papillary muscle involvement in only about 30% of the cases. The relative frequency of papillary muscle involvement is approximately the same in the three infarction type groups.

Isolated papillary muscle infarction was seen in two cases (9%), and this seems to be the normal frequency. Andersen & Hansen (1972) found 4 cases out of 40 (10%) and Andersen & Agdel (1976) found one case out of 16 (6%). Erhardt (1974) did not find any in his material of 84 cases.

Right ventricular wall. Infarction of the right ventricular wall was seen in only 18% of the AMI cases. In patients dying from AMI after treatment in a coronary care unit it is much more common. Erhardt (1974) found right ventricular involvement in approximately 40% of all patients, but also that, in accordance with our findings, it was uncommon in patients with subendocardial infarctions. As they are the most frequent in our material this difference in frequency of right ventricular involvement is not unexpected.

CONCLUSION

In this paper the results of heart-autopsies in cases of sudden death are presented. The main conclusions are that AMI is less common than it appears from the study of death certificates and that infarction located subendocardially is the most frequent, which indicates that this is the primary infarction type and that 76% of the AMI's were non-thrombotic.

I wish to thank Dr Viggo Andersen, medical department, Sundby Hospital, and Dr Jørgen Løng, Medico-legal Institute, Copenhagen, for the assistance provided.

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TABLE 3 *Salient Autopsy Findings in Relation to Degree of Stenosis*

	Number of coronary arteries more than 50% narrowed			
	0	1	2	3
Number of observations	21	24	19	22
Old infarctions	0	13	13	16
Myocardial fibrosis	9	9	13	16
Heart weight > 500 g	5	7	11	9
Old and/or fresh thrombi	0	9	4	8

Isolated papillary muscle infarction was seen in two cases.

Infarction of the right ventricular wall was seen in 8 cases (18%) with no significant differences in the relative frequency between the infarction types.

Infarction of the septum was observed in 13 cases (29% $m.p. = 0.69$ not significant), most frequently together with combined infarctions.

Myocardial fibrosis was found in a total of 47 cases. It was equally frequent in the AMI-group and in the non AMI-group and also showed no relation to infarction type. The frequency of heart weights greater than 400 and 500 grams has been determined in all groups, but heart weight does not show any significant relation to AMI or to infarction type.

The degree of stenosis has been compared to the frequency of other observations in Table 3. Old infarctions were only seen when at least one coronary artery was narrowed to more than 50% of the lumen. Old infarctions were also seen more frequently when three coronary arteries showed stenosis than when only one did but these differences are not significant. Myocardial fibrosis was seen with a higher frequency the higher the degree of stenosis, and the difference in the relative frequency of myocardial fibrosis when one artery ■

narrowed (39%) is significantly lower ($P < 0.01$) than the relative frequency when all three were narrowed (73%).

There was no relation between the degree of coronary stenosis and heart weight or the occurrence of thrombi.

DISCUSSION

Patients. The present findings were all on hearts from patients who died suddenly but there was no information as to whether the death was expected or not. The sex and age distribution of the material however is very close to the distribution found by Bekker & Grünfeld (1975). They from one month's death certificates from Denmark found that 299 males (mean age: 66 years) and 17 females (mean age: 70 years) had died suddenly and unexpectedly. The male proportion was 84% which compares well with our 0.64 - as do the mean ages (our study: males, 68.3 years, females 70.2 years). The frequency of AMI is different in the two studies however as Bekker & Grünfeld found that 70% of the cases died with AMI whereas we found AMI in only 52% of the cases. This difference is probably explained by the fact that Bekker & Grünfeld's material is based on death certificates and not on autopsy findings.

Types of Infarctions. The relative frequency of transmural combined and subendocardial infarctions found in our study is compared to the findings of Andersen & Apgal (1976), Andersen & Horn (1972) and Erhardt (1974) in Table 4. Combined infarctions are found with an equal relative frequency in all four studies. Transmural infarction is the least frequent type in our study and also in the study of Andersen & Apgal (1976) but the most frequent in Erhardt's (1974) material. Subendocardial infarction on the other hand, is the most common in the first two studies and the least common in the last mentioned. The results obtained by Andersen & Hansen 1972 occupy an intermediate position.

TABLE 4 *Relative Frequency of Infarction Types Found in Different Authors*

	Types of Infarctions			Total
	Transmural	Combined	Subendocardial	
Our study	11%	29%	60%	45
Andersen & Apgal (1976)	13%	13%	74%	15
Andersen & Hansen (1972)	28	25	47	40
Erhardt, L. H. (1974)	58%	23%	19%	84

MEGAKARYOCYTES IN PULMONARY BLOOD VESSELS

1. Incidence at Autopsy: Clinicopathological Relations especially to Disseminated In- travascular Coagulation.

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Aabo, K. & Hansen, K. B. Megakaryocytes in pulmonary blood vessels. I. Incidence at autopsy clinicopathological relations especially to disseminated intravascular coagulation. *Acta path. microbiol. scand. Sect. A*, 86: 285-291, 1978.

In a study of 365 consecutive hospital autopsies and 21 forensic autopsies in previously healthy individuals, who had died suddenly, intravascular megakaryocytes (MK) were found in 94% (95% of the hospital series and 67% of the forensic series). The average value in the forensic series was 4 MK per cm² of lung tissue (0-16 MK per cm²) with an estimated maximum value of 18 MK per cm² ($p < 0.0005$). The average value in the hospital series was 37 MK per cm² (0-765 MK per cm²). Defining 25 MK per cm² as the upper limit for normal counts in the lung, 35% (126/365) had increased values, 9% (32) having more than 100 MK per cm². The MK observed were mature forms, mostly naked nuclei, and in one case only megakaryoblasts were seen. MK were predominantly found in the lungs and very rarely in any of the other organs examined (spleen, liver and kidney). A correlation which is statistically significant to a high level was found between an increased number of pulmonary MK and manifest intravascular coagulation ($p < 0.001$), acute infections ($p < 0.001$), liver insufficiency ($p < 0.001$), bleedings ($p = 0.007$), cancer ($p = 0.008$), shock ($p = 0.013$) and fever ($p = 0.060$). We also found a statistical correlation between low values of pulmonary MK and leukemia ($p = 0.007$). The diseases and clinical conditions which are positively correlated to an increased number of pulmonary MK (except bleedings) are known to be correlated to intravascular coagulation too. We therefore assume that consumption coagulopathy is the common pathogenic mechanism explaining the increased number of pulmonary MK except in the case of bleedings where extrinsic platelet loss is the most likely mechanism.

Key words: Megakaryocytes, intravascular coagulation, microthrombosis.

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Received 2 Jan. 77 Accepted 6 Feb. 78

Intravascular megakaryocytes (MK) is a physiological phenomenon, first described by Aschoff in 1893. The lung is the organ where extramedullary MK are most commonly found, and the incidence in other organs is directly correlated to the incidence in the lung (Brill and Halpern 1948). Different methods of quantitation of pulmonary MK have been used, however, such as counts per slice (Smith and Butcher 1952), number of positive cases

(Seebach and Kernohan 1952), counts per field of vision (120 x, Moucheski 1966), counts per cm² (Sharnoff and Kim 1958 and Bettendorf and Meyer-Breding 1974), and only one paper (Smith and Butcher 1952) includes a satisfactory normal material.

Positive correlations between MK incidence in pulmonary capillaries and certain diseases such as acute infections (Aschoff 1893, Smith and Butcher 1952, Seebach and Kernohan 1952, Sharnoff and

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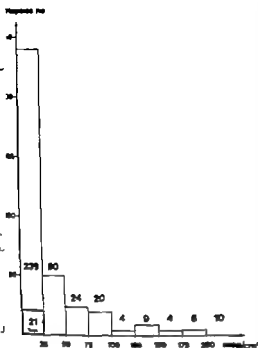


Fig. 1 Incidence of pulmonary megakaryocytes in 365 hospital autopsies □ and 21 forensic autopsies ■

(50/365) had values of 25–50 MK per cm² 13% (44/365) had values of 51–100 MK per cm² and 9% (32/365) had values of more than 100 MK per cm² (Fig. 1).

The intravascular MK were nearly always mature forms, including naked nuclei. In one case only (nonmetastatic adenocarcinoma of the coecum postoperatively complicated with pleural empyema and a large intrahepatic haematoma) with a MK

count of 100 per cm² of lung tissue, we found cells resembling megakaryoblasts (Fig. 2). The MK were most often found in the lung capillaries, occasionally in small pulmonary arteries and very rarely in small veins.

Microthrombosis Of the hospital series 25% (92/365) had grade 3 or grade 4 when screening PTAH stained sections alone, 22% (80) had grade 3 or grade 4 in the lungs, 6% (21) in the spleen, 2% (6) in the liver and only 0.3% (1) in the kidney. Because of the unspecificity of the staining of fibrin and platelets, grade 3 or grade 4 microthrombosis must be present in both stains (PTAH and PAS) to diagnose MIC, according to our experience. In our material 51 had MIC; 11 DIC and 40 LIC. MIC was found in 13% (48/365) of the lungs, 3.5% (13/365) of the spleens, 0.5% (2/365) of the livers and 0.3% (1/365) of the kidneys. We found a correlation which is statistically significant at a high level ($p \leq 0.001$) between an increased number of MK in the lungcapillaries and MIC (Table 1).

Relations between pulmonary MK counts and various clinico-pathological data are demonstrated in Table 2 and Table 3.

Infectious Fever Leucocytosis Infections were found in 179 cases, mostly pneumonia, and 43% (76) had an increased number of pulmonary MK, 28% (49) having more than 50 MK per cm². The figure is significantly increased ($p \leq 0.001$). Fever on the day of death was found in 60 cases and 47% (28) had an increased number of pulmonary MK ($p = 0.060$). Leukocytosis was found in 106 cases out of which 42% (44) had an increased number of pulmonary MK.

Liver insufficiency was found in 33 cases (curbosis and metastases with ascites) and 67%



Fig. 2 Megakaryocytic cells in lungcapillaries. A. cytoplasmic megakaryocytes and naked nuclei. B. megakaryoblast.

Kim 1958 Moschinski 1966 and Bettendorf and Meyer Breiting 1974) cancer (Smith and Butcher 1952 and Sharnoff and Kim 1958) thromboembolic diseases (Smith and Butcher 1952 and Sharnoff and Kim 1958) and postoperative states (Sharnoff 1959) are known as well as positive correlations between intravascular coagulation and infections (Saldeen 1967 McKay 1965-1968 Harms and Lehmann 1969 Skjorten 1970 Nalbandian et al 1971 and Mjyre Jensen et al 1972) cancer (McKay 1965-1968 Skjorten 1970 Nalbandian et al 1971 and Mjyre Jensen et al 1972) liver insufficiency (McKay 1965-1968 Nalbandian et al 1971 and Wintrobe 1974) and shock (McKay 1965-1968 Harms and Lehman 1969 and Nalbandian et al 1971). To our knowledge no one has ever studied the relation between MK counts in the lungcapillaries and intravascular coagulation and so far a satisfactory explanation to the elevation of the number of circulating MK in the above mentioned conditions has not been given.

Studying retrospectively a selective autopsy material with pathoanatomically verified disseminated intravascular coagulation we found very high MK counts in the lungcapillaries. Four of the patients had died in renal insufficiency in connection with shock (bilateral renal cortical necrosis) and three had died from meningococcal septicæmia. Waterhouse-Friedenreichs syndrome (necrosis and suffusions in the skin plus bleedings in the adrenal glands). This encouraged us to perform this study. In order to estimate the incidence at autopsy of intravascular MK and to study the correlation between intravascular pulmonary MK and microthrombosis as well as the correlation to several clinical conditions in more detail than previously done 365 consecutive hospital autopsies and 21 selected forensic autopsies were studied.

MATERIAL AND METHODS

Specimens were collected from 365 consecutive hospital autopsies performed during a one year period (1975-1976). Furthermore, specimens were collected from 21 selected autopsies from the department of forensic medicine (previously completely healthy individuals in whom death had occurred suddenly and without any injury of the thorax). From each autopsy in the hospital series specimens from lungs, liver, spleen and kidneys were obtained. In the forensic series only specimens from the lungs were sampled. The tissue specimens were taken from areas without pathological changes such as emphysema, atelectasis, metastases, pneumonia and major vessels and bronchi which would give a misleading estimation of the area of the histologic section. The tissues were fixed in 4% neutral, phosphate buffered formaldehyde solution, embedded in paraffin

and cut at about 7 microns. The sections were stained with haematoxylin (HE), periodic acid-Schiff reaction (PAS) and phosphotungstic acid reaction (PTAH). The stained sections, usually 1-2 or 3, were screened blindly for the presence of microthrombi and scored after a semiquantitative method (adapted from Mjyre Jensen et al 1972): 0, no thrombi; 1, 1 thrombi per slice; 2, thrombi in less than 10% of vessels; 3, thrombi in 10-50% of the vessels; 4, thrombi in more than 50% of the vessels. Grade 1's were designated manifest intravascular coagulation (MIC). If only one organ was involved we designated as local intravascular coagulation (LIC) and if two more organs were involved as disseminated intravascular coagulation (DIC). The grading was based on PTAH-PAS stains and performed by one of the authors (K) while the other (K.B.H.) screened blindly the sections for MK, i.e. without knowledge of the grade of microthrombosis or clinical pathological findings at time of screening.

An analysis of the screenings was performed in and in duplicate by the two authors and showed 100% correspondence. The screening for MK was performed with 250 \times magnification. The area of the tissue used were directly measured in square centimeters. Platelet count per cm² were used as a rough correlation to grade of microthrombosis. Case reports as well as autopsy reports were studied retrospectively and the following conditions were tested for correlation to MK counts: age, sex, time of bed to death, temperature on the day of death, sediment rate, leukocytosis, anaemia, thrombocytopenia, O₂ PP%, thromboembolism, anticoagulant treatment, infections, shock, bleedings, blood transfusion, acute diseases, cytostatical treatment, liver insufficiency, diabetes mellitus. A rank sum test relating the parameters to the rest of the series (e.g. tests of life against non infections) was used.

RESULTS

The MK counts in the 21 forensic autopsies of previously healthy individuals (9 females and 12 males with an age range from 1 day to 71 years) less than 1 year: 7 between 1 year and 20 years; between 20 years and 50 years and 5 more than 50 years) varied from 0 to 16 MK per cm² of tissue, with an average value of 4 MK per cm². The estimated maximum value was found to be 18 MK per cm² ($p < 0.005$). The MK counts in the hospital autopsies varied from 0 to 765 MK per cm² of lung tissue, with an average value of 37 MK per cm². Sections with unspecific slightly elevated values seems to be «normal» in hospital autopsies and we therefore designated 25 MK per cm² (50% of 18 MK per cm²) as the upper normal limit in the hospital series. MK were found in the capillaries in 95% (348/365), and 35% (126/365) had values of more than 25 MK per cm².

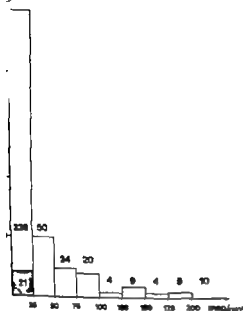


Fig. 1. Incidence of pulmonary megakaryocytes in 365 postnatal autopsies □ and 21 forensic autopsies ■.

40/365) had values of 25–50 MK per cm², 13% (4/365) had values of 51–100 MK per cm² and 1% (32/365) had values of more than 100 MK per cm² (Fig. 1).

The intravascular MK were nearly always mature forms, including naked nuclei. In one case only (nonmetastatic adenocarcinoma of the coecum, intraoperatively complicated with pleural empyema and a large subhepatic haematoma) with a MK

count of 100 per cm² of lung tissue, we found cells resembling megakaryoblasts (Fig. 2). The MK were most often found in the lung capillaries, occasionally in small pulmonary arteries and very rarely in small veins.

Microthrombosis. Of the hospital series 25% (92/365) had grade 3 or grade 4 when screening PTAH stained sections alone, 22% (80) had grade 3 or grade 4 in the lungs, 6% (21) in the spleen, 7% (6) in the liver and only 0.3% (1) in the kidney. Because of the unspecificity of the staining of fibrin and platelets, grade 3 or grade 4 microthrombosis must be present in both stains (PTAH and PAS) to diagnose MIC according to our experience. In our material 51 had MIC, 11 DIC and 40 LIC. MIC was found in 13% (48/365) of the lungs, 3.5% (13/365) of the spleens, 0.5% (2/365) of the livers and 0.3% (1/365) of the kidneys. We found a correlation which is statistically significant at a high level ($p \leq 0.001$) between an increased number of MK in the lungcapillaries and MIC (Table 1).

Relations between pulmonary MK counts and various clinico-pathological data are demonstrated in Table 2 and Table 3.

Infections. Fever. Leucocytosis. Infections were found in 179 cases, mostly pneumonia, and 43% (76) had an increased number of pulmonary MK, 28% (49) having more than 50 MK per cm². The figure is significantly increased ($p \leq 0.001$). Fever on the day of death was found in 60 cases, an 47% (28) had an increased number of pulmonary MK ($p = 0.060$). Leukocytosis was found in 106 cases out of which 42% (44) had an increased number of pulmonary MK.

Liver insufficiency was found in 33 cases (cirrhosis and metastases with icterus) and 67%



Fig. 2. Megakaryocytic cells in lungcapillaries. A: cytoplasmic megakaryocyte and naked nucleus. B: megakaryoblast.

TABLE 1 *Relation between Number of Pulmonary Megakaryocytes (MK) and Microthrombosis Statistical Correlation to Manifest Intravascular Coagulation (MIC)*

		Numbers of autopsies, grouped after number of pulmonary MK per cm ²				total
		0-25 abs. (%)	26-50 abs. (%)	51-100 abs. (%)	>100 abs. (%)	
DIC	present	3 (77)	3 (27)	3 (27)	2 (19)	11
LIC	present	17 (42)	8 (20)	8 (20)	7 (18)	43

MIC	present	20 (38)	11 (22)	11 (27)	9 (18)	51
MIC	not present	219 (70)	39 (12)	33 (11)	23 (7)	314

MIC (manifest intravascular coagulation) grade 3 and 4 microthrombosis in both PAS and PTAH stained sec.
DIC (disseminated intravascular coagulation) grade 3 and 4 microthrombosis in two or more organs

LIC (localized intravascular coagulation) grade 3 and 4 microthrombosis in one organ.

* * The correlation between increased number of pulmonary MK and MIC is statistically highly significant ($p < 0.001$)

TABLE 2 *Relations between Number of Pulmonary Megakaryocytes (MK) and Various Clinical and Pathological Conditions Statistical Correlation to Increased Number of Megakaryocytes*

		Numbers of autopsies, grouped after number of pulmonary MK per cm ²				total	Statistical correlation (p value)
		0-25 abs. (%)	26-50 abs. (%)	51-100 abs. (%)	>100 abs. (%)		
Infections	not present	136 (72)	23 (12)	19 (10)	8 (6)	186	$p < 0.001$
	present	103 (57)	27 (15)	25 (14)	24 (14)	179	
Fever	not present	127 (69)	22 (12)	19 (10)	17 (9)	185	$p = 0.060$
	present	32 (53)	10 (17)	12 (20)	6 (10)	60	
Leucocytosis	not present	51 (72)	7 (10)	7 (10)	6 (8)	71	$p = 0.128$
	present	62 (58)	15 (14)	15 (14)	14 (14)	106	
Liver insuff. ficiency	not present	228 (69)	41 (12)	38 (11)	25 (8)	332	$p < 0.001$
	present	11 (33)	9 (27)	8 (18)	7 (22)	33	
Bleedings	not present	220 (66)	46 (14)	38 (11)	29 (9)	333	$p = 0.003$
	present	8 (40)	4 (20)	5 (25)	3 (15)	20	
Malignancy	not present	154 (68)	32 (14)	24 (10)	7 (8)	227	$p = 0.003$
	present	59 (55)	17 (16)	18 (17)	14 (12)	108	
Shock	not present	119 (69)	36 (12)	31 (11)	24 (8)	290	$p = 0.013$
	present	30 (49)	11 (18)	13 (21)	7 (12)	61	
Thrombo- embolism	not present	189 (67)	42 (15)	32 (11)	20 (7)	283	$p = 0.211$
	present	50 (60)	8 (10)	12 (15)	12 (15)	82	

7) had an increased number of pulmonary MK (≤ 0.001)

Haemorrhage was found in 20 cases and 60%

2) had an increased number of pulmonary MK

(≤ 0.003) Cases with major haemorrhages (5) all had more than 50 MK per cm^2

Malignant neoplastic diseases were found in 108

cases and 45% (49) had an increased number of pulmonary MK ($p = 0.008$)

Shock was present in 61 cases out of which 51%

1) had an increased number of pulmonary MK

($p = 0.013$) The cases were 9 with ileus shock, 7

with septic shock, 39 with cardiogenic shock and 6

with bleedings

Thromboembolism was found in 82 cases out of which 40% (32) has an increased number of pulmonary MK, 57 had thrombosis in the femoral vein with emboli in the lung. Although a tendency towards an increased number of pulmonary MK was found, the figure was not statistically significant.

Leukaemia Thrombocytopenia PP% Cytostatical treatment Leukaemia was found in 18 cases and none of them had an increased number of pulmonary MK ($p = 0.007$) and neither had any of the 13 cases with thrombocytopenia ($p = 0.024$). Decreased PP% was present in 20 cases and only 10% (2) had an increased number of pulmonary

TABLE 3 Relations between Number of Pulmonary Megakaryocytes (MK) and Various Clinical and Pathological Conditions. Statistical Correlation to Low Number of Megakaryocytes

		Numbers of megaplates, grouped after number of pulmonary MK per cm^2				total	Statistical correlation (p value)
		0-25 abs (%)	26-50 abs (%)	51-100 abs (%)	>100 abs (%)		
Leukaemia	not present	221 (64)	50 (14)	44 (13)	32 (9)	347	$p = 0.007$
	present	18 (100)	0 (0)	0 (0)	0 (0)	18	
Thrombocytopenia	not present	14 (70)	3 (16)	2 (10)	0 (0)	19	$p = 0.024$
	present	13 (100)	0 (0)	0 (0)	0 (0)	13	
PP%	normal	4 (45)	2 (22)	1 (11)	2 (22)	9	$p = 0.072$
	decreased	18 (90)	0 (0)	1 (5)	1 (5)	20	
Cytostatical treatment	not given	219 (64)	49 (14)	43 (13)	31 (9)	347	$p = 0.072$
	given	70 (88)	1 (4)	1 (4)	1 (4)	23	
Age	≤ 50 years	8 (33)	5 (21)	5 (21)	6 (25)	24	$p = 0.012$
	> 50 years	231 (68)	45 (13)	39 (11)	26 (8)	341	
Sex	males	124 (66)	27 (14)	21 (11)	16 (9)	188	
	females	115 (65)	23 (13)	23 (13)	16 (9)	177	
Diabetes	not present	219 (66)	45 (14)	40 (12)	29 (8)	333	
	present	20 (63)	5 (16)	4 (12)	3 (9)	32	
Blood transfusion	not given	201 (65)	44 (14)	40 (13)	27 (8)	312	
	given	28 (72)	3 (8)	4 (10)	4 (10)	39	
Sedimentation rate	normal	18 (67)	3 (11)	3 (11)	3 (11)	27	
	increased	67 (67)	12 (12)	12 (12)	9 (9)	101	
Time in bed	≤ 7 day	96 (63)	23 (15)	18 (12)	16 (10)	153	
	> 7 days	142 (67)	27 (13)	26 (12)	16 (8)	211	
Anaemia	not present	74 (65)	13 (12)	12 (11)	13 (12)	112	
	present	54 (65)	14 (17)	8 (9)	8 (9)	84	

MK ($p=0.07$) Cytostatical treatment had been given in 23 cases and only 12% (3) had an increased number of pulmonary MK ($p=0.07$) The relation between the number of MK in the lung capillaries and leukaemia as well as other haematological disorders will be considered in a separate paper

Miscellaneous An association between an increased number of pulmonary MK and age less than 50 years was found though not significant, and no correlation was found between an increased number of pulmonary MK and sex diabetes mellitus, anaemia blood transfusion, increased sedimentation rate, and time in bed before death

DISCUSSION

The average value in the forensic series («normal material») in this study was 4 MK per cm² of lung tissue with an estimated maximum value of 18 MK per cm² This figure is identical to that of *Smith and Butcher* (1952) who found an average value of 5.1 MK per section of approximately 1 cm² In the hospital series the average number was 37 MK per cm² of lung tissue 35% (126) having increased values more than 25 MK per cm² We found MK in 95% (348/365) of the cases which is in agreement with *Gornes* (1924) *Brill and Halpern* (1948) and *Smith and Butcher* (1952) *Smith and Butcher* like *Sharnoff and Kim* (1958) found average values of 10–14 MK per cm² and the latter found maximum values of 30–40 MK per cm² It is difficult to explain the differences as their material includes the same groups of diseases as ours. High counts of pulmonary MK (as ours 765) have been reported by *Bettendorf and Meyer Breiting* (1974) who in 6 cases found 60–1200 MK per cm² of lung tissue and by *Moschinski* (1966) who in 9% (34/382) found more than 3 MK per field of vision (120 \times) *Moschinski* also like us found increased number of pulmonary MK in 34% of their autopsies Extrapulmonary intravascular MK have been dealt with by many authors, the spleen being the organ most often involved though to a much lesser extent than the lungs. In our study no attempt was made to quantitate the extrapulmonary MK. In this study a high grade of microthrombosis was found in 25% (92) when using PTAH stain for fibrin alone, 51 cases, however were positive both in PTAH and PAS stained sections The organ most often involved in MIC was the lung (13%) and thereafter the spleen (3.5%) the liver (0.5%) and the kidney (0.3%) These figures correspond to other investigations *Saldeen* (1967) found intravascular coagulation in 18% of 301 lungs, especially when associated with pneumonia, bronchitis and infarction *Harms and Lehman* (1969) in 18% of

519 lungs *Mahre Jensen et al* (1972) found red microthrombosis in 12% of hospital autopsies, but only 0.5–5.0% had microthrombosis of a type which in our study is called MIC. A correlation which is statistically significant at a high level was thus found between increased number of pulmonary MK and several clinico-pathological conditions among these MIC as defined here Stimulation of the megakaryocytopoiesis and thrombocytopoiesis by platelet depletion is known to increase the maturation rate of MK and to increase the number of circulating MK (*Efte et al* (1968) *Odell et al* (1969) and *de Gabrielle et al* (1967)) Intravascular coagulation with consumption of platelets will stimulate the thrombocytopoiesis and by doing so institute an increase in the number of circulating MK. Depression of the bone marrow on the other hand will result in a decreased number of circulating MK, and in this study we found a statistically significant correlation between a low number of MK and leukaemia as well as cytostatical treatment and thrombocytopoiesis.

The correlation between increased number of pulmonary MK and fever as well as leucocytosis is probably explained by the correlation between increased number of pulmonary MK and acute infections also found in this study This correlation was first demonstrated by *Aschoff* in 1893 and later by *Smith and Butcher* (1952), who in 30 cases with infections found a 3–4 times increase in the MK number The positive correlation between increased number of pulmonary MK and other states of diseases (thromboembolism cancer liver insufficiency shock and haemorrhage) as demonstrated in this study is also in accordance to previous investigations.

Intravascular coagulation is an intermediary mechanism of disease (*McKay* (1965)) and has been found correlated to such conditions as acute infections malignant neoplastic diseases, shock, postoperative states and cirrhosis. Thus there is an amazing concordance between the conditions which are correlated to intravascular coagulation and the conditions which are correlated to an increased number of pulmonary MK. This observation and the fact that manifest intravascular coagulation as defined in this study is statistically correlated to an increased number of pulmonary MK at a high level of significance, indicate that consumption coagulopathy might be the common pathogenetic mechanism explaining the increased number of pulmonary MK in the above mentioned states of diseases. We found that an increased number of pulmonary MK occurs in several conditions (acute infections, liverinsufficiency cancer shock and thromboembolism) and therefore is

not specific for certain diseases, but these clinical conditions which are known to be correlated to consumption coagulopathy obviously lead to an increased MK release from the bone marrow and thereby an increased number of pulmonary MK as a result of stimulation of the megakaryocyto-thrombopoiesis by the consumption of platelets

We thank Dr. med. V. Gregersen, Institute of Forensic Medicine, Århus Kommunehospital, University of Århus, for kind assistance in collecting the normal material

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MEGAKARYOCYTES IN PULMONARY BLOOD VESSELS

2. Relations to Malignant Haematological Diseases especially Leukaemia

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Hansen K B and Aabo K. Megakaryocytes in pulmonary blood vessels. 2. Relations to malignant haematological diseases especially leukaemia. *Acta path microbiol scand Sect A*, 86: 293-295, 1978.

In a study of 71 autopsies in patients with malignant haematological disorders (55 leukaemias and 16 multiple myelomas) we found an increased number of megakaryocytes in the lung capillaries in only one of 55 cases of leukaemia (43 acute and 12 chronic leukaemias) with a mean value of 3 megakaryocytes per cm^2 . The incidence of pulmonary megakaryocytes in 16 cases of multiple myeloma was identical to that in an unselected, consecutive series of hospital autopsies. The discrepancy between the increased megakaryocytopoiesis and previously reported high number of circulating megakaryocytes in chronic myeloid leukaemia, and the few megakaryocytes in the pulmonary blood vessels of histological sections of autopsy specimens is discussed.

Key words: megakaryocytes, leukaemia

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Received 2 ix 77 Accepted 6 ii 78

Since Oelkefen and Naegeli both in 1914 found a positive correlation between the incidence of circulating megakaryocytes and chronic myeloid leukaemia, there has been a special interest in the relations between the malignant haematological disorders and the quantitative and morphological aspects of circulating megakaryocytes. The positive correlation has since been verified by several investigators (Di Guglielmo (1916-1923), Almon (1922), Sampson (1971), Estévez *et al* (1971), Gorbia *et al* (1972, 1973), Maldonado (1974) and Albrecht *et al* (1974). Perri (1928), however, found no correlation between chronic myeloid leukaemia and an increased number of circulating megakaryocytes.

The incidence of megakaryocytes in lung capillaries in cases of malignant haematological disorders has been dealt with by few investigators only.

Recently we (Aabo and Hansen (1977)) found a significant correlation between low numbers of pulmonary megakaryocytes and leukaemia (18 cases), defining 25 megakaryocytes per cm^2 as the upper normal limit. In order to estimate the incidence at autopsy of intravascular pulmonary megakaryocytes in malignant haematological diseases 71 selected autopsies were studied.

MATERIAL AND METHODS

71 autopsies with leukaemia and multiple myeloma performed at the Institute of Pathology Århus Amtssygehus, in a 6 year period (1970-1976) were selected. 55 had leukaemia (43 acute and 12 chronic) and 16 had multiple myeloma. Haematoxylin-eosin stained sections of lungs from the files were screened for megakaryocytes. 5 megakaryocytes per cm^2 of lung tissue were defined as the upper normal limit for the number of pulmonary megakaryocytes (Aabo and Hansen (1977)).

TABLE 1 *Relations between Number of Pulmonary Megakaryocytes per cm² and Malignant Haematological Disorders*

	Numbers of autopsies, grouped after number of pulmonary meg. per cm ²				Total
	0-75 abs (%)	26-50 abs. (%)	51-100 abs (%)	>100 abs (%)	
A.M.L.	29 (97)	1 (3)	0 (0)	0 (0)	30
A.L.L.	8 (100)	0 (0)	0 (0)	0 (0)	8
A.L. others	5 (100)	0 (0)	0 (0)	0 (0)	5
A.L. total	42 (98)	1 (7)	0 (0)	0 (0)	43
C.L. total	12 (100)	0 (0)	0 (0)	0 (0)	12
A.L. + C.L.	54 (98)	1 (2)	0 (0)	0 (0)	55
M.M.	10 (63)	4 (25)	1 (6)	1 (6)	16

AML = acute myeloid leukaemia ALL = acute lymphoid leukaemia AL = acute leukaemia. CL = chronic leukaemia, myeloid and lymphoid MIM = multiple myeloma

RESULTS

Of 43 autopsies with acute leukaemia (30 myeloid, 8 lymphoid and 5 with other forms of acute leukaemia) only one (2%) had an increased number of pulmonary megakaryocytes, 29 per cm². The mean value of the acute leukaemia material was 3 megakaryocytes per cm². None of 12 autopsies with chronic leukaemia (myeloid and lymphoid) had an increased number of pulmonary megakaryocytes, the mean value being 3 megakaryocytes per cm². 38% (6/16) of the cases of multiple myeloma had an increased number of pulmonary megakaryocytes, the mean value being 24 per cm² (range: 0-108 per cm²) (Table 1).

DISCUSSION

This study confirms our preliminary findings that leukaemia is correlated to a low number of megakaryocytes in the lung capillaries. This correlation is remarkable, because most of the patients with leukaemia had suffered from severe infections and several from septicaemia, conditions which are known to be correlated to increased numbers of pulmonary megakaryocytes. A correlation between leukaemia and a low number of pulmonary megakaryocytes was also found by Moschinski (1916) who in 28 cases of leukaemia (17 myeloid and 11 lymphoid) found elevated values in only 3 cases. Furthermore the 3 cases showed only slightly elevated values. Goronzi (1924) described 3 cases of leukaemia in which only one megakaryocyte was found in the lung capillaries and Petri (1928) reports 3 cases of chronic myeloid leukaemia (only one had megakaryocytes in the lung capillaries, 25 per cm²). 5 cases of chronic lymphoid leukaemia (none had megakaryocytes in the lung

capillaries) and 4 cases of acute leukaemia (pulmonary megakaryocyte counts, 0-45 per cm²).

Although megakaryocytes in the lung capillaries in cases of leukaemia are few many investigators have found a very high number of circulating megakaryocytes in blood from patients with chronic myeloid leukaemia (Hoegeli (1914), Oelshelger (1914) and DiGuglielmo (1916-1920)). An increased number of circulating megakaryocytes in other myeloproliferative disorders has been reported (Wiskul (1948) Chan *et al* (1971), Voigt (1971), Saksela *et al* (1971) Smith *et al* (1973), Estess *et al* (1973) and Bettendorf and Meyer Breiting (1971)). Minot (1922) and Wiskul (1948) assumed that a puffy an acute exacerbation of the disorders.

In addition to the correlation of an increased number of megakaryocytes in the blood recent publications have shown a correlation between megakaryocyte morphology and myeloproliferative diseases. Abnormal small dwarf megakaryocytes have been found in chronic myeloid leukaemia (Trautman (1961) Harker *et al* (1969) Saksela (1971), Lagerlof and Franzen (1972) Goronzi *et al* (1972-1973) Queisser *et al* (1973), Smith *et al* (1973) Albrecht *et al* (1974), Papern (1974) and Maldonado (1974)) and very large megakaryocytes in polycythaemia (Harker *et al* (1969), Lagerlof and Franzen (1972) and Albrecht *et al* (1974)). Trautman introduced the term «microkaryocytes» for the small megakaryocytes found in chronic myeloid leukaemia, and Albrecht *et al* used the term for megakaryocytes from 150-800 square microns, which is equivalent to a diameter of approximately 10-25 microns.

The reason why we and others did not find an increased number of megakaryocytes in the lung capillaries in cases of chronic myeloid leukaemia, although a correlation exists between an increased

number of circulating megakaryocytes and chronic myeloid leukaemia, might be the small size of the megakaryocytes (=mikrokaryocyten) in chronic myeloid leukaemia. The very small megakaryocyte 10-25 microns in diameter might not be found in the lung capillaries and those being stained might be impossible or at least very difficult to distinguish from other cells in the lung artery due to their size and partly due to their low rate of polyploidy and thereby intensity of nuclear staining. Furthermore, the fact that most cases of chronic myeloid leukaemia terminates in blastosis and are treated with cytostatics which both lead to depression of the megakaryocytopoiesis, might contribute to explaining the correlation found between chronic leukaemia and a low number of pulmonary megakaryocytes at autopsy.

The cases of multiple myeloma show an incidence of pulmonary megakaryocytes, which is equivalent to the incidence in a series of 365 consecutive hospital autopsies (Aabo and Hansen (1977)). Although one would expect a decrease in number of pulmonary megakaryocytes due to depression and displacement of the bone marrow by myelomas, we found no such decrease. It is likely therefore, that the replacement of the bone marrow in multiple myeloma is not so extensive that the capacity of megakaryocytopoiesis is severely disturbed.

As a conclusion intravascular pulmonary megakaryocytes are found in a lower number than is normal in autopsies of leukaemia, acute as well as chronic. The incidence and number of pulmonary megakaryocytes in autopsies with multiple myeloma are identical with those in other non-haematological diseases.

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THE REACTION OF THE VASCULAR PATTERN OF THE HYPERTROPHIED MYOCARDIUM TO INCREASED CARDIAC VOLUME LOAD

A Microangiographical Study

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Carlsson, S, Ljungqvist, A, Törnelling, G & Unger, G. The reaction of the vascular pattern of the hypertrophied myocardium to increased cardiac volume load. A microangiographical study. *Acta path microbiol scand Sect. A*, 86: 297-301 1978.

Myocardial stereo-microangiography was performed on 30 rats after swimming exercise for one or two periods with and without a previous operation for production of aortic stenosis. In 8 rats with aortic stenosis no swimming exercise followed and 10 more rats served as normal controls. Swimming exercise resulted in a higher degree of cardiac hypertrophy than aortic stenosis. Both types of cardiac hypertrophy resulted in vascular adaptive reactions in the myocardium. The adaptive reaction to swimming exercise was found to be persistent, at least to some extent, and to be of use if the exercise was resumed. The adaptive alterations in aortic stenosis were further modified by a superimposed period of exercise although the murine capillary vasculature only showed a slight and focal increase in density.

Key words: Myocardium, hypertrophied, vascular pattern, microangiography.

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Accepted as submitted 8.8.78

In earlier works we observed an increased density of capillaries in stereo-microangiograms of hearts subjected to increased volume load (swimming exercised rats) but not in microangiograms of hearts subjected to increased pressure load (rats with aortic stenosis and arterial hypertension) (Ljungqvist & Unger 1972). In the latter hearts a number of spiralling arteries and arterioles were found, a feature which was also observed in swimming exercised rats provided that they had rested for some time after the exercise.

The swimming-induced vascular alterations were judged to be the result of growth of myocardial arteries and arterioles and neoformation of capillaries in the hypertrophied myocardium. This interpretation was supported by findings in further experiments in which ³H-thymidine was injected into rats with increased cardiac volume load

(swimming exercise) and pressure load (aortic stenosis) followed by light and electron microscope autoradiography (Mandache *et al* 1972, 1973 and Ljungqvist & Unger 1973).

In more recent studies similar experimental methods provided evidence that swimming exercise will not induce any significant neoformation of myocardial capillaries if the heart is already rendered hypertrophic by aortic stenosis (Ljungqvist *et al* 1976) and that the myocardial capillaries formed during swimming exercise will be available for further use when exercise is resumed after a period of rest (Carlsson *et al*).

The aim of the present investigation was to find out whether swimming exercise would modify the myocardial vascular pattern induced by (i) a previous period of swimming exercise and (ii) aortic stenosis.

MATERIAL AND METHODS

For y-eight Sprague Dawley rats with an initial weight of 180–200 g were used. The animals were housed in cages, 3–5 in each cage. They were fed a standard diet containing 0.5% sodium chloride and tap water *ad libitum*. The animals were divided into three main groups.

Group 1

Twenty animals were exercised in a 180 × 80 × 90 cm large tank at a water temperature of about +28°C. The animals swam 1 hour per day 6 days per week. They were divided into two subgroups.

- Ten animals which were killed after 4 weeks of swimming exercise followed by a resting period of 8 weeks.
- Ten animals which were killed after having exercised for 4 weeks, rested for 4 more weeks and exercised again for another 4 week period.

Group 2

- Eight animals which were killed 6 weeks after the production of aortic stenosis according to the method of Bernak (1952).
- Ten animals which were swimming-exercised for 4 weeks after having rested for 2 weeks following the production of aortic stenosis.

Group 3

Ten untreated rats served as normal controls.

On termination of the experimental periods each rat was anaesthetized with ether and weighed. The sternum was then split open and the heart exposed. Five ml of a 10 per cent suspension of fine grain barium sulphate (Micropaque) were injected into the left atrium. The animal died during this injection, after which a catheter was inserted in a retrograde direction into the ascending aorta. The vessels to the head and front extremities were ligated and micropaque infused via the catheter for about 60 minutes. The infusion pressure was gradually elevated from 30 to 110 mm Hg. During the initial phase of the infusion irregular heart activity with ventricular contractions was recorded in most animals.

On termination of the infusion the heart was removed and fixed in 10% formaldehyde for 24–28 hours. Specimens were then prepared for stereomicroangiographic examinations according to previously-described methods (Ljungqvist 1963). Briefly these included the production of 3–4 mm thick transverse slices of the ventricular segment from the sino-ventricular level to the apex. These slices were then embedded in a mixture of beeswax and paraffin, after which they were cut in 100–800 μ thick blocks. The blocks were exposed by stereoscopic technique in a fine focus x ray tube and on a fine-grained photographic emulsion (Kodak spectroscopic plates type 649-0). The microangiograms were studied in a specially constructed stereo-microscope.

RESULTS

Table 1 shows that a significant increase in heart weight/body weight ratio occurred in all experimental groups with the exception of group 2B.

Normal Vascular Pattern

The normal vascular pattern has been described earlier (Ljungqvist & Urge 1972). In brief it includes large subepicardially located arteries which send major vessels into the myocardium. The vessels are of two types, a-vessels, which are split up at sharp angles into 3–7 large branches, and b-vessels which depart from the coronary arteries at angles of 50–90° and continue without division to the subendocardial capillary plexus (cf. Esser *et al.* 1966; Farrer Brown & Hartman 1967). The normal pattern includes occasional spiralled arteries and a striking parallelism of the vessels forming the capillary networks.

Group 1A (Exercise Followed by Rest)

When compared with the rats of group 1B the rats showed two important features. (a) Spiralled arteries were more frequent, and spiralled arteries and capillaries were seen (Fig. 1). (b) no increase in density of the capillary vasculature was noticed.

Group 1B (Repeated Exercise)

The septum arteries and their branches are dislocated towards the lumen of the right ventricle. No spiralled vessels were observed. The capillary networks appeared denser than normal, and preserved parallelism of their vessels (Figs 2 and 3).

TABLE 1. Heart Weight × 1000/Body Weight (Mean) Rats with One (1A) and Two (1B) Periods of Swimming Exercise and in Rats with Aortic Stenosis without (2A) and with (2B) a Superimposed Period of 5 weeks exercise for comparison. One Group of Normal Control Rats is Included (3)

Group of rats	No of rats	Ratio (mean ± SD)
1A	10	5.43 ± 0.49
1B	10	5.45 ± 0.40
2A	8	5.10 ± 0.94
2B	10	4.68 ± 0.63
3	10	4.65 ± 0.21

Significantly different from controls

(0.01 < p < 0.05)

Significantly different from controls (p < 0.001)



Fig 3 Micro-angiogram from left ventricle wall of hypertrophied rat heart (repeated exercise), showing a denser capillary network than normal (cf Fig 2) $\times 90$

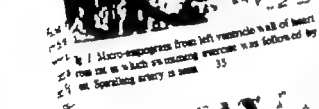


Fig 4 Micro-angiogram from left ventricle wall of heart from rat in which a running exercise was followed by an. Spontaneous artery is seen $\times 35$



Fig 4 Micro-angiogram from left ventricle wall of hypertrophied rat heart (acute stenosis), showing segmental filling defects, which extend from the up- to the endocardial surface $\times 15$



Fig 5 Micro-angiogram from left outside wall of control rat heart, showing normal capillary pattern $\times 40$

Group 2 A (Aortic Stenosis)

The arteries and arterioles appeared wider than in the control animals. There was a marked irregularity and poor parallelism in the capillary pattern, with numerous short intercapillary anastomoses at all levels but no definite change in the density of the capillary network. Spiralling arteries were observed in the left ventricle wall and septum as were large numbers of spiralling precapillary and capillary vessels. Segmental filling defects (Ljungqvist & Unge 1972) were frequent throughout the left ventricle wall (Fig. 4).

Group 2 B (Aortic Stenosis Followed by Exercise)

No changes were found in the septum arteries. There was a marked irregularity and poor parallelism in the capillary pattern. No spiralled arteries, arterioles and capillaries were found and no segmental filling defects. The density of the capillary networks was not increased.

DISCUSSION

In previous investigations we have shown that swimming exercise and aortic stenosis will result in cardiac hypertrophy in the rat, the degree of hypertrophy induced by swimming being the most pronounced (Ljungqvist & Unge 1973). We have also found that the degree of hypertrophy induced by aortic stenosis will not be further increased by a superimposed period of swimming, the ultimate degree of hypertrophy thus being less than that produced by swimming alone (Ljungqvist *et al.* 1976). In the present investigation largely the same observations were made, the sole exception being the absence of a detectable degree of hypertrophy in the group of rats in which the production of aortic stenosis was followed by swimming exercise. This can be explained by the fact that the ratio figures of the present material may well have been influenced by variable degrees of contrast filling of the various hearts, the figures therefore being very approximate.

In the present investigation the previously reported microangiographical alterations of the hypertrophied myocardium were again demonstrated (Ljungqvist & Unge 1972). The pattern in the rats with repeated periods of exercise was not different from that previously observed in rats examined directly after a single period of exercise. The existence of spiralled vessels in hearts from rats which have been resting after a period of exercise has previously been regarded as a feature of decreased functional demands on vessels induced to grow during physical exercise. The disappearance of spiralling when exercise is resumed indicates that

these vessels were incorporated into the normal adaptation to the renewed increased demands. It therefore appears that myocardial vascular adaptation to physical exercise can, at least to some extent, persist for a certain period of time and be of use when the exercise is resumed.

In swimming-exercised rats with aortic stenosis no increased capillary density was noted. This supports previous observations suggesting that hearts hypertrophied from increased pressure are less liable to respond to an increase in workload by a capillary neoformation than are normal hearts (Ljungqvist *et al.* 1976). A certain, though focal, capillary proliferation appears to take place, however, since the segmental filling defects characteristic for hearts from rats with aortic stenosis (Ljungqvist & Unge 1972) were abolished by a superimposed period of swimming exercise.

The fact that spiralling of myocardial vessels induced by aortic stenosis was abolished by a superimposed period of swimming is difficult to evaluate. The probable explanation is that swimming induced a further, although not recent, heart enlargement, resulting in straightening of spiralled vessels.

This study was supported by the Swedish Medical Research Council (project no. B75-12X 716).

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ULTRASTRUCTURAL AND HISTOCHEMICAL OBSERVATIONS ON SEROUS OVARIAN CYSTADENOMAS

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Klemi, P. J. & Nevalainen, T. J. Ultrastructural and histochemical observations on serous ovarian cystadenomas. *Acta path. microbiol. scand. Sect. A*, 86: 303-312, 1978.

One hundred and twelve serous ovarian cystadenomas (44 benign, 33 tumours of borderline malignancy and 33 malignant tumours) were investigated with histological and modern histochemical methods. Electron microscopy was applied to 9 benign, 6 borderline and 5 malignant tumours. The epithelium of the benign serous cystadenomas contained ciliated and associated columnar cells, which sometimes were edematous («pale cells»), and smaller basal cells. The large Golgi apparatus with small secretory vesicles and occasional larger secretory granules was located in the apical cytoplasm. There were numerous pinocytotic vesicles at the basal plasma membrane. The extracellular matrix in the cystic lumen and the matrix on the apical epithelial border consisted of material with vic-glycols (1,2-hydroxy) groups stained with sulpho- and carbocyanine. With increasing degree of malignancy the number of ciliated cells diminished and were not found in the malignant epithelium. The number of nuclei and the amount of carbocyanine with sialic acid residues increased with increasing degree of malignancy. However there were no single consistent ultrastructural feature which could be considered as a reliable criterion for malignancy of these tumours in the absence of invasive growth. The findings support the view that serous ovarian cystadenomas are of coelomic origin.

Key words: Serous ovarian tumours, cystadenoma of ovary, histochemistry, electron microscopy, mucosubstances.

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Accepted as submitted 15 78

Serous cystadenomas constitute the most common group of tumours of the human ovary (Kew & McKay 1960, Bennington *et al.* 1968 and Auer *et al.* 1971), and they belong to the «common epithelial tumours of the ovary» in the WHO's classification (Sera *et al.* 1973). Serous cystadenomas are histologically divided into benign and malignant tumours, and tumours of borderline malignancy according to the proliferative activity and invasive growth of the epithelial cells. The histologic picture of the benign tumours is well defined, but the tumours of borderline malignancy and the malignant tumours are often very difficult to distinguish from each other if invasive growth is not found. Serous cystadenomas secrete much and it has been proposed that the use of histochemical methods in

differentiating the tumours may be helpful (Long & Summers 1969). Various ultrastructural differences such as increased irregularity of nuclei and decreased amount of plasma membrane interdigitations with increasing malignancy of the serous tumours, have been found. Cells were present in benign tumours and in tumours of borderline malignancy but were not found in malignant tumours (Gondos 1971). Another study (Blumstein 1976) could not confirm a number of these findings.

Benign serous ovarian cystadenomas are totally harmless after their resection. Tumours of borderline malignancy may give rise to distant implants, but even so the prognosis is good. The malignant serous cystadenomas on the other hand have the poorest prognosis of all common epithelial tumours of the ovary and thus establishing the right

diagnosis for a given tumour may be of superior importance (Santenissson & Kottmeier 1968 and Aure *et al* 1971).

The purpose of the present work was to study the ultrastructure of the serous ovarian cystadenomas and to characterize histochemically the epithelial mucins of these tumours. This was done in order to facilitate the differential diagnosis of serous ovarian cystadenomas of varying degrees of malignancy and to confirm the histogenetic origin of these tumours.

MATERIAL AND METHODS

The material consisted of 112 cases which were grouped according to the WHO's classification (Serov *et al* 1973) into 46 benign, 33 borderline and 33 malignant serous ovarian cystadenomas. Paraffine sections were stained with various histochemical methods as presented elsewhere (Klemi 1978) for the characterization of the epithelial mucosubstances and argyrophil and argentaffin cells in the tumours.

Small pieces from 20 tumours including 9 benign, 6 borderline and 5 malignant serous cystadenomas were processed for electron microscopy as described elsewhere (Klemi & Hevalainen 1978).

RESULTS

The cases, mean ages of the patients having serous ovarian tumours and the occurrence of psammoma bodies are shown in Table 1. Goblet, argyrophil or argentaffin cells, which are typical of mucinous ovarian cystadenomas (Klemi 1978), were not found in the serous tumours. Hyaluronidase digestion did not alter the staining reactions of the epithelial mucosubstances. The ultrastructure and histochemistry of epithelial mucosubstances in the serous tumours are dealt with in turn.

Benign Serous Cystadenomas

Ultrastructure There were 3 cell types in the epithelium lining the cyst walls and their papillary projections in the benign serous cystadenomas.

TABLE 1 The Mean Ages (SD: Standard Deviation) of the Patients Having Serous Ovarian Cystadenomas of Different Degrees of Malignancy and the Number of Tumours Containing Psammoma Bodies

Type of tumour	No of cases	Mean age \pm SD	No of tumours with psammoma bodies
Benign	46	50.6 \pm 18.9	14
Borderline	33	48.1 \pm 13.6	7
Malignant	33	56.5 \pm 10.3	10

ciliated, nonciliated and basal cells (Fig. 1). The main type was a columnar or cuboidal, apical cell which had short microvilli with poorly developed short rootlets on the luminal surface. The second type was similar but in addition to the microvilli there were cilia on the apical cell surface. The cytoplasm of both ciliated and nonciliated was sometimes edematous. These slightly swollen cells correspond to the apical cells seen in the light microscope specimens. The basal cells were located on the basal lamina between the other epithelial cells.

The ultrastructure of the ciliated and nonciliated cells was rather similar. There were well developed desmosomes and tight junctions between adjacent epithelial cells. The lateral plasma membranes were smooth or slightly interdigitating. The oval nuclei were relatively large with some deep invaginations in the nuclear membrane. One (rarely two) distinct nucleolus was present (Table 2). The cytoplasm contained numerous round or slightly elongated mitochondria, some dense bodies (most probably lysosomes), many free ribosomes and one or two profiles of the Golgi complex with small vesicles (Fig. 2). The endoplasmic reticulum was poorly developed. The mitochondria were often greatly swollen. Myelin figures especially in connection with the mitochondria were found sporadically. The mitochondrial swelling and the myelin figures were most probably caused by suboptimal tissue preservation. Glycogen granules were not found. Microfilaments and some microtubules were scattered throughout the cytoplasm. The shafts of the cilia were covered by protrusions of the plasma membrane. They contained one central and nine peripheral pairs of microtubules. There were numerous microfilaments extending from the rootlets of the cilia into the apical cytoplasm (Fig. 2).

The basal cells were triangular or cubic in shape and had only few organelles in their cytoplasm. The apical cell surfaces did not reach the lumen (Fig. 1). There were numerous pinocytotic vesicles in the plasma membranes over the basal lamina (Fig. 4). The ground substance of the connective tissue under the basal lamina was homogeneous without any identifiable structures. There were normal-looking fibroblasts, collagen fibers and amorphous material in the deeper parts of the connective tissue stroma (Fig. 1).

Histochemistry The staining reactions for intracellular mucins were mostly negative. However both in the ciliated and nonciliated cells there were occasional cytoplasmic granules which stained well with the periodic acid-Schiff method with and without previous diastase digestion (DPAS and



Fig. 1 An electron micrograph of a benign serous ovarian cystadenoma. The epithelium consists of low columnar or cylindrical ciliated (CL), nonciliated (NC) and triangular basal cells (BC). The cytoplasm is adenomatous giving a vacuole cell appearance (P) to some ciliated and nonciliated cells. A number of the superficial cells bud into the lumen (CB). Beneath the basal lamina (B) there are fibroblasts, collagen fibers and anisophase material of the connective tissue. Stained with uranyl acetate (UA) and lead citrate (Pb) $\times 5,500$.

PAS). This material corresponded to the dense cytoplasmic bodies, which stained well with the periodic acid-silver methenamine method (PASVI) at the ultrastructural level and most probably were lysosomes. The apical border of the ciliated and nonciliated cells contained 1,2-hydroxyl groups and

both sulphy- and carboxymucin (Fig. 4). Only one of the 46 benign tumours contained carboxymucin with sialic acid residues. The mucin in the lumen consisted of 1,2-hydroxyl groups mixed with ac-mucin as did that on the apical border of the epithelial cells.

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Histochemistry The staining reactions for intracellular mucins were mostly negative. However, in the ciliated and nonciliated cells there were occasional cytoplasmic granules which stained with the periodic acid-Schiff method with or without previous diastase digestion (dPAS).

TABLE 2 The Number of Nucleoli in 20 Serous Ovarian Cystadenomas of Different Degrees of Malignancy

Type of tumor	No. of cases	No. of nuclei counted	Percentage of nuclei containing 1 2 3 or more nucleoli			
			1	2	3	more than 3
benign	9	2700	89	10	1	—
borderline	6	1700	83	15	2	—
malignant	5	1300	65	24	6	3

contained more nucleoli than the nuclei of the benign cystadenomas (Table 2). A few cells contained some glycogen granules. The basal lamina was continuous as that of the benign tumours.

Histochemistry Some cells contained cytoplasmic granules which stained well with the dPAS and PAS methods as was seen also in the benign serous cystadenomas. The rest of the cytoplasm contained neither neutral nor acid mucins. The apical epithelial border stained well with the High-Iron-Diamine-Aldrich Blue pH 2.5 (HID-AB pH 2.5) method indicating the presence of both sulpho- and carboxymucin. The total amount of mucin in the

apical border was greater than that found in this border in the benign tumours. In 6 tumours the carboxymucin was digested to a high degree with salivase.

Malignant Serous Cystadenomas

Ultrastructure The main cell type in the malignant serous tumours was the nonciliated cell. It was columnar, cuboidal, round or oval in shape (Fig. 6). There were more differentiated areas in the malignant cystadenomas, in which the epithelium was quite similar to that in the tumours of borderline malignancy. In the poorly differentiated areas the cells were more irregular in shape. The

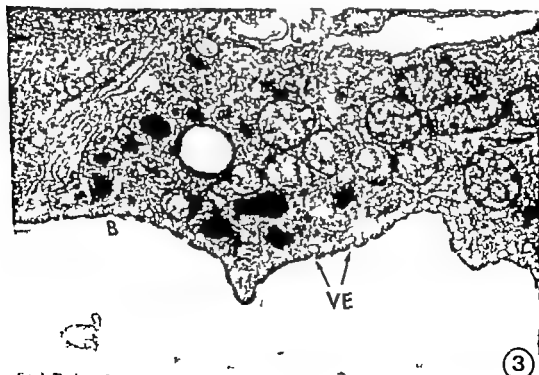


Fig. 1 The base of an epithelial cell in a benign serous cystadenoma. Basal lamina (B). There are numerous poecyotic vesicles (VE) in the plasma membrane. UA + Pb. $\times 30,000$.



Fig. 2 The apical part of a ciliated cell in a benign serous cystadenoma. The cytoplasm contains elongated or rootlets often swollen mitochondria, many polyribosomes (PR) and a prominent Golgi complex (G). The cilia are anchored to the apical cytoplasm by rootlets (RO) (N), nucleus. UA + Pb $\times 16\ 800$

Serous Cystadenomas of Borderline Malignancy

Ultrastructure The cells in the epithelium of the serous tumours of borderline malignancy showed some tendency of piling up. Most of the epithelial cells were nonciliated and low columnar or cuboidal in shape (Fig. 5). Ciliated and «pole cells» were present too, but they were not as numerous as in the

benign cystadenomas. The lateral plasma membranes were slightly interdigitating or smooth, and in the membranes in the benign tumours, the contained junctional specializations (desmosomes, tight junctions). The cytoplasmic ultrastructure of the epithelial cells in these tumours did not differ from that in the benign tumours. The nuclei

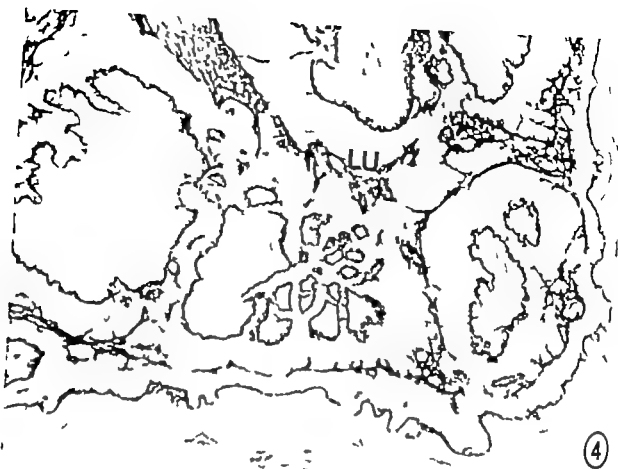
Fig 4 A light micrograph of a benign serous ovarian cystadenoma. The secretory material in the lumen (LU) and on the apical epithelial border is mainly sulphomucin rich, appears black in this picture. Stained with High-magnification Alcian blue at pH 2.5 $\times 140$

Fig 5 An electron micrograph of a serous ovarian cystadenoma of borderline malignancy. The epithelium consists of nonciliated (NC) and ciliated (CT) cells which show some tendency of piling up. UA = P.b. $\times 3,400$

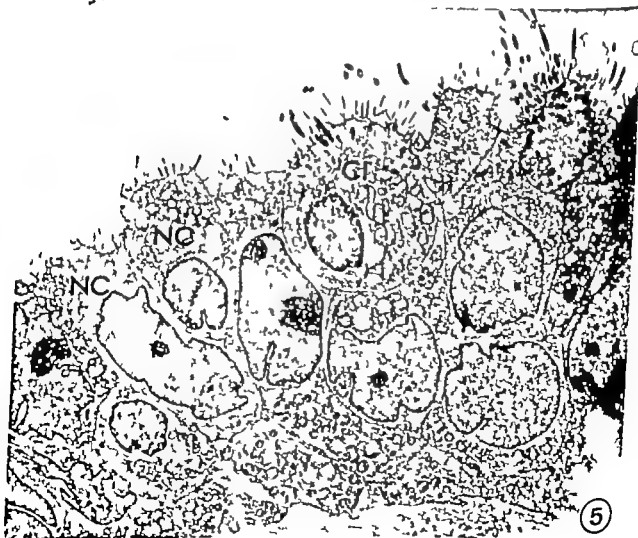
lateral plasma membranes were relatively smooth with few desmosomes. The number of nucleoli was larger than in the benign or borderline tumours (Table 2). There were some ciliated cells in the better differentiated areas, where the tumours resembled the borderline tumours. However in the poorly differentiated areas of the malignant cystadenomas there were no ciliated cells. The tumour cells contained the same organelles as the cells in the less malignant tumours. The Golgi apparatus was



Fig 6 An electron micrograph of malignant serous ovarian cystadenoma. The tumour consists of nonciliated epithelial cells of various shapes and sizes with irregular nuclei and large nucleoli. Some of the superficial cells bud into the lumen (CB). 1: A P.b. $\times 3,600$



4



5

4 A light micrograph of a benign serous ovarian adenoma. The secretory material in the lumen (LU) on the apical epithelial border is mainly sulphomucin and appears black in this picture. Stained with High-magnification Alcian blue at pH 2.5 $\times 140$.

5 An electron micrograph of a serous ovarian adenoma of borderline malignancy. The epithelium consists of nonciliated (NC) and ciliated (CI) cells which show some tendency of piling up. UA = Pb $\times 3,400$.

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Fig 6 An electron micrograph of malignant serous ovarian cystadenoma. The tumour consists of nonciliated epithelial cells of various shapes and sizes with irregular nuclei and large nucleoli. Some of the superficial cells bud into the lumen (CB). UA = Pb $\times 3,400$.

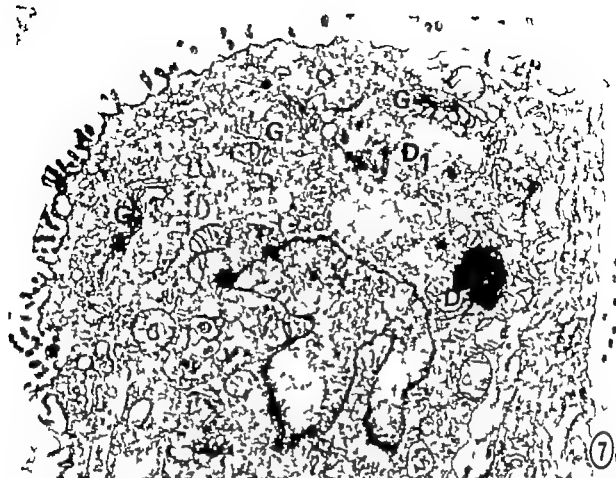


Fig 7 Malignant serous cystadenoma. There are numerous profiles of the Golgi complexes (G) in the apical cytoplasm. There are relatively poorly staining homogeneous, large granules (D₁) closely associated with the Golgi cisternae and a dense more heterogeneous body (D₂) in the cytoplasm. UA + Pb. $\times 11,400$

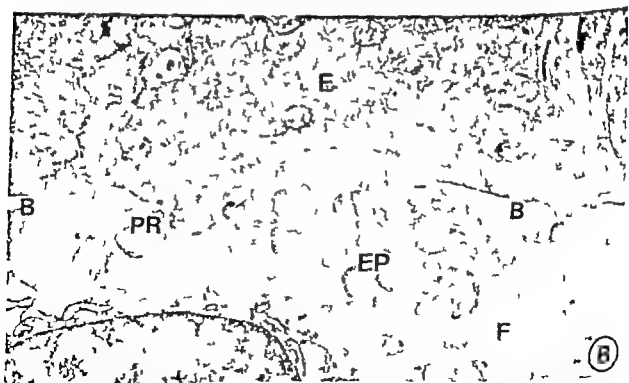


Fig 8 An electron micrograph of malignant serous cystadenoma. The basal lamina (B) is disrupted and epithelial projections (EP) penetrate deep into the subepithelial connective tissue. Epithelial cell (E) and fibroblast (F). UA Pb $\times 12,000$

entire in some cells (Fig. 7). In some cells there are relatively large, homogeneous and poorly staining granules closely associated with the Golgi apparatus, in addition to denser heterogeneous cytoplasmic granules (Fig. 7). The latter were most probably secondary lysosomes whereas the former represent secretory granules. Both type of vesicles stained well with the PASM-method. The cells contained glycogen granules as well. In every tumour the basal lamina was sometimes ruptured with epithelial projections deep into the connective tissue, indicating invasive growth (Fig. 7). The basal plasma membrane contained less vacuolated vesicles than that in less malignant tumours.

Staining reactions. The staining reactions for neutral and acid mucins were generally negative in the cells, however as in the nonmalignant cystadenomas, there were in the tumour cells some cytoplasmic granules which stained with dPAS and PAS. These responded to the cytoplasmic dense bodies which stained intensely with the PASM-method at the ultrastructural level. Most probably the majority of these dense bodies were lysosomes, although the possibility that some of them represented secretory granules (Fig. 7) could not be ruled out by the histochemical staining techniques used in this study. The apical cell border stained with varying intensity with the HID-AB pH 2.5 method, but mostly similarly to that of the borderline tumours. Six tumours contained mucin with a large amount of sialic acid residues. On the other hand there were some areas where no mucin could be found.

DISCUSSION

The observations of the present study showed that the epithelium of the serous ovarian cystadenomas is made up of low columnar or cuboidal cells with or without cilia, and of undifferentiated smaller basal cells. With increasing malignancy increased histologically the nonciliated cells showed a tendency to pile up, and at the same time became more irregular in shape. The epithelium of the benign serous cystadenomas contained many ciliated cells, but these cells were found only sporadically in borderline tumours and were absent in the malignant epithelium. However there were better differentiated areas in the malignant tumours, which resembled those in the borderline and benign cystadenomas. These areas contained ciliated cells. Therefore the absence of ciliated cells in a serous cystadenoma may indicate malignancy of the tumour but the presence of ciliated cells alone cannot be regarded as an indication of the benign

nature of such tumours as postulated earlier by Gondos (1971). In the borderline tumours there were areas where the benign epithelium gradually transformed into a borderline one, and similarly in the malignant tumours the borderline type of epithelium gradually transformed into a malignant one. Such transitions indicate that all serous ovarian cystadenomas have the same origin, and they develop into more malignant ones by cellular proliferation from their benign counterparts. Thus it is possible to find ciliated cells in the malignant serous cystadenomas, as shown in two earlier studies (Roberts *et al.* 1970 and Blaauw *et al.* 1976). The increased nuclear irregularity and membrane interdigitations were found to be indications of increased malignancy of the serous ovarian cystadenomas (Gondos 1971) but the present study could not confirm these changes as reliable criteria for malignancy as pointed out earlier by Blaauw *et al.* (1976). The number of nucleoli increased with increasing malignancy. This is a reliable indication of malignancy as has been observed also earlier (Long & Sommers 1969).

The vesicles at the basal plasma membranes serve a nutritive function. Such vesicles were found also in the cells of cystadenofibromas of the ovary (Papadakis & Bellis 1975). The basal lamina was continuous in the benign and borderline tumours, but disrupted in the malignant tumours. This phenomenon is a reliable and constant finding for differentiating borderline and malignant tumours from each other. The distribution of psammoma bodies in the serous cystadenomas was similar to that observed before (Uure *et al.* 1971). The presence or absence of the psammoma bodies did not correlate with the malignancy of the tumours.

Some cells in every serous tumour of different malignancy contained granular intracytoplasmic material with 1,2-hydroxyl groups. Most of this material was shown to correspond to the dense cytoplasmic granules at the ultrastructural level, most probably lysosomes. Both ciliated and nonciliated cells contained prominent Golgi complexes with small vesicles, indicating secretory function. Some large homogeneous secretory granules were closely associated with the Golgi apparatus. They did not show any substructure and thus differed ultrastructurally from the secretory granules in the epithelial cells of the mucinous ovarian cystadenomas (Klemi & Avelainen 1978). The amount of sulphomucin or carboxymucin in the apical borders of the epithelial cells did not differ significantly in tumours of different degrees of malignancy. Sialic acid residues seemed to increase slightly with increasing malignancy but this finding was not constant enough to be used as a diagnostic aid in

differentiating the borderline and malignant tumours from each other as has been proposed (Long & Sommers 1960)

All of the cell types (ciliated and nonciliated columnar or cuboidal cells and basal cells) seen in the serous ovarian cystadenomas resembled closely the cells of the fallopian tube (Fredricsson & Björkman 1962). Ciliated cells are numerous also in the epithelia of the endocervical canal and ovarian cystadenofibromas (Laguens *et al* 1967 Cernobilsky *et al* 1974 and Papadaki & Beilby 1975). The epithelia of the fallopian tube, the endometrium and the endocervical canal originate from the Müllerian duct which develops early in fetal life by invagination of the coelomic epithelium. This coelomic epithelium covers also the ovarian cortex and can give rise to the serous cystadenomas directly or via the formation of inclusion cysts. (von Humers 1965 Roberts *et al* 1970 Gondos 1971 Fox *et al* Langley 1976 and Radisavljevic 1977)

The conclusion that can be made from the present observations is that the disruption of the basal lamina indicating invasion of a serous ovarian cystadenoma, is a constant and reliable feature which helps in distinguishing borderline and malignant tumours from each other. The absence of ciliated cells, the increased number of nucleoli, and the presence of malleic acid residues in a serous tumour with cellular and nuclear atypia have only secondary importance as indicators of malignancy. If invasive growth is not observed in a given section one should make more sections to exclude the existence of such growth.

This work was supported by a grant from the Emil Aaltonen Foundation. The authors thank Miss P. Anttila, Miss E. Saarinen, Mrs A. Suoranta, Mr M. Lehtimäki and Miss M. L. Arvola for skilful technical assistance.

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PANCREATIC B-CELL SENSITIVITY TO ALLOXAN IN VIVO

*A Study of Antagonizing Compounds, Serum Inorganic Phosphate
and Acid Base Balance*

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Boquist, Lennart. Pancreatic B-cell sensitivity to alloxan *in vivo*. A study of antagonizing compounds, serum inorganic phosphate and acid-base balance. *Acta path. microbiol. scand. Sect. A*, 86: 313-318, 1978.

Serum glucose, inorganic phosphate, acid-base balance and islet morphology were studied in mice subjected to different kinds of treatment before alloxan administration. In preceding studies, pre-treatment with D-glucose, D-mannose, D-fructose, sodium lactate and NaHCO_3 , but not with D-galactose, was found to protect against alloxan toxicity. In this study alloxan antagonism was found by pre-treatment with L-leucine, but not with L-arginine or NaH_2PO_4 . Blood analyses, carried out 10 minutes after injection of the test compounds, disclosed that the serum inorganic phosphate concentration was decreased in mice given D-glucose, D-mannose, D-fructose, sodium lactate, NaHCO_3 , L-leucine, and L-arginine, but was not affected in those treated with D-galactose, and was increased in those receiving NaH_2PO_4 . With the exception of L-arginine, blood pH was increased in the groups which exhibited decreased serum inorganic phosphate concentration, pH was not affected in mice treated with D-galactose, L-arginine and NaH_2PO_4 . Alterations in inorganic phosphate and hydrogen ion concentration may affect the B-cell sensitivity to alloxan (pH - pH hypothesis).

Key words: Pancreatic B-cells, alloxan, sensitivity *in vivo*, inorganic phosphate, acid-base balance.

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Received 13.1.78 Accepted 24.2.78

In recent works in this laboratory attention has been paid to factors which affect the B-cell sensitivity to alloxan *in vivo* in mice. Since pre-treatment with 1,25-dihydroxycholecalciferol (1,25-DHCC) or parathyroid hormone (PTH) was found to increase the alloxan sensitivity (Boquist *et al.* 1977), and since differences were observed in the cation precipitation, investigated by pyroantimonate technique and X-ray microanalysis, between alloxan treated and control mice (Boquist 1977a), it was thought of interest to study calcium and inorganic phosphate (Pi), extracellularly and, if possible, intracellularly in relation to the development of alloxan diabetes.

The present work is concerned with serum Pi and was undertaken against the background of the preceding studies, to see whether the previously demonstrated protection against alloxan toxicity by pre-treatment with D-glucose, D-mannose, D-fructose (Boquist 1977), sodium lactate and NaHCO_3 (Boquist 1978) is associated with any alteration in the serum concentration of Pi. In addition, the B-cell sensitivity to alloxan, and serum Pi were studied after pre-treatment with L-leucine, L-arginine and NaH_2PO_4 . Parameters for acid-base balance were also investigated since they are of importance in the evaluation of alterations in serum Pi concentration, and since metabolic alkalosis in a

preceding work (Boquist 1978) was found to decrease the B-cell sensitivity to alloxan.

MATERIAL AND METHODS

Animals Treatment and Blood Sampling

The animals were male non-diabetic adult C57BL/Ksl +/+ mice from a local stock kept under standard laboratory conditions at a constant temperature of 22 °C. They had free access to water and a standard laboratory ration containing 1.0% calcium and 0.75% phosphorus. The contents of vitamin D₃ were approximately 600 IU per 100 g diet. The animals were deprived of food for 24 hr before the first injection and until after the blood sampling at the 4 hr observation time. The substances used for treatment were injected intraperitoneally under ether anaesthesia.

The following experimental groups were used: I Saline (controls); II D-glucose; III D-mannose; IV D-fructose; V D-galactose; VI Sodium lactate; VII NaHCO₃; VIII L-leucine; IX L-arginine; X NaH₂PO₄; and XI Alloxan (solely). In experiments on alloxan sensitivity alloxan was given 10 min after the test compound in groups I, VIII, IX and X.

The following doses (aqueous solutions) were given: saline - 50 ml/kg b.w.; sugars - 100 mg/kg b.w.; sodium lactate - 16 mmol/kg b.w.; NaHCO₃ - 20 ml/kg b.w. of a 5% solution; L-leucine - 4 mmol/kg b.w.; L-arginine - 400 mg/kg b.w.; NaH₂PO₄·H₂O - 150 mg/kg b.w.; and alloxan - 200 mg/kg b.w.

D-glucose was obtained from British Drug Houses Ltd. England. D-mannose, D-fructose, D-galactose, sodium lactate, L-leucine and L-arginine were from Sigma Chemical Co., St. Louis, Mo. U.S.A. Alloxan monohydrate was obtained from Eastman Kodak Co., Rochester, N.Y., U.S.A.

Blood for glucose determination was obtained by cutting the tip of the tail, whereas blood for determination of serum Pi, pH, pCO₂ and standard bicarbonate was obtained by heart puncture at 22 °C. Purely arterial blood could not be obtained in sufficient amounts by this method. To avoid venous admixture, only blood with a minimum pO₂ of 6.0 kPa was used for further analyses.

Determination of Serum Glucose, Pi, pH, pCO₂, pO₂ and Standard Bicarbonate

Blood glucose was assayed by the glucose oxidase method using Glucose (AB Kabi, Stockholm, Sweden) on animals from groups I, VIII, IX, X and XI before the injections, and at the following predetermined intervals after alloxan administration: 1, 2 and 4 hr and 1, 2, 3 and 4 days.

The serum concentration of Pi, pH, pCO₂, pO₂ and standard bicarbonate was determined 10 min after treatment (corresponding to the interval used between pre-treatment and alloxan administration both in this and in preceding studies) in groups I, II, III, IV, V, VI, VII, VIII, IX and X. The blood for these analyses was in every group sampled about 8 a.m.

Serum Pi was determined with the aid of a Technicon autoanalyser (Technicon Instr. Co., Ardsley, N.Y.,

U.S.A.). The pH, pCO₂, pO₂ and standard bicarbonate were assayed with standard technique using a Radiometer ABL 1 (Radiometer, Copenhagen, Denmark). The analyses were carried out immediately after blood sampling.

Light and Electron Microscopy

Some animals from groups VIII, IX and X were killed 4 and 48 hr following mg alloxan injection, at which the pancreas was removed and most of it was used for light microscopic study whereas the rest was processed for electron microscopy.

Specimens for light microscopy taken only at 4 hr were fixed in Bouin's fluid and stained with special techniques for islet studies. Specimens for electron microscopy taken both at 4 and 48 hr were fixed in 2.5% glutaraldehyde in 0.34 M cacodylate buffer, pH 7.4 followed by post fixation in osmium tetroxide. Embedding was carried out in Epon 812 and the sections stained with uranyl blue were used for identification of the islets. The thin sections were stained with uranyl acetate and lead citrate and then examined in a Siemens Elmiskop 101.

RESULTS

Blood Glucose

The results of the blood glucose determination are given in Table 1. A typical triphasic blood glucose response to alloxan was found in the pre-treated mice (group XI), and in the mice which received L-arginine (group IX) and NaH₂PO₄ (group X) 10 min before alloxan administration. The blood glucose concentration was not significantly altered in the control animals injected with saline (group I) or in mice treated with L-leucine (group VIII) 10 min before alloxan injection.

Serum Pi, pH, pCO₂ and Standard Bicarbonate

The results of the laboratory analyses are given in Table 2. The serum concentration of Pi in controls was 2.56 ± 0.08 mmol/l (mean \pm S.E.M.). In comparison with this there was decreased level of Pi in the groups treated with glucose, D-mannose, D-fructose, sodium lactate, NaHCO₃, L-leucine and L-arginine. Serum Pi was not significantly altered in the group given galactose but was increased in the group treated with NaH₂PO₄.

The hydrogen ion concentration was slightly increased in the groups treated with D-glucose, mannose, D-fructose and L-leucine, and markedly elevated in those given sodium lactate and NaHCO₃ but not significantly altered in those treated with galactose, L-arginine or NaH₂PO₄. Increased standard bicarbonate was observed in the groups given sodium lactate and NaHCO₃. The standard bicarbonate was not affected in the other experiments.

TABLE 1 Blood Glucose Concentration (mg/100 ml) in Starved Mice before (0 hr) Pre-treatment, and at Different Intervals after Treatment with Alloxan or Saline, Injected 10 min after Pre-treatment. The Number of Observations is Given in Brackets

		Blood glucose concentration (Mean \pm S.E.)									
Pre-treatment	Treatment	hours			days						
		2	3	4	1	3	5				
No	Saline	75.2 (67)	51.8 (347)	5.9 (206)	48.3 (47)	14.77 (107)	6.36 (110)	4.96 (104)	10.4 (76)	10.4 (76)	4.6 (34)
I	Alloxan	44.4 (61)	59.2 (516)	5.9 (76)	41.8 (276)	101.9 (276)	109.4 (276)	107.3 (276)	96 (195)	96 (195)	6 (6)
I	L-tyrosine	36 (5)	149.0 (87)	143.8 (87)	47 (6)	6.07 (113)	8 (2)	15.07 (113)	341.9 (162)	16.27 (162)	322.2 (137)
I	NaH ₂ PO ₄	56.4 (87)	176.0 (87)	160.6 (87)	63.6 (87)	317.3 (737)	7.37 (2)	15.07 (113)	315.1 (1747)	17.47 (1747)	24.2 (1137)
No	Alloxan	57 (307)	15.3 (1204)	1.2 (103)	49.3 (633)	119 (1030)	238.9 (1470)	533.3 (157)	346.7 (1137)	41.37 (1137)	41.37 (1137)

The animals were fed again after blood sampling at 4 hr. This explains the blood glucose elevation at 1 day in the controls and in the groups which were insensitive to alloxan, and might have contributed to the hyperglycaemia in the other groups.

groups. No significant difference was found between the groups in pCO₂ values.

Light and Electron Microscopy

Light microscopy of the pancreas disclosed typical B-cell lesions (Boquist 1977; Boquist *et al.* 1977) 48 hr following alloxan injection in non-pre-treated mice (group XII), and in mice pre-treated with L-tyrosine (group IX) or NaH₂PO₄ (group X). Islets composed of unaffected B-cells, on the other hand, were encountered as mice treated with L-leucine (group VIII). A few B-cells with slight

cytoplasmic degeneration could be observed in a few islets from these mice.

Electron microscopy showed typical B-cell lesions (Boquist 1977; Boquist *et al.* 1977) at both observation times in groups IX, X and XI (Fig. 1). The islets from group VIII were either normal or had occasional B-cells with mitochondrial lesions at 4 hr and a few B-cells with mitochondrial lesions, cytoplasmic vacuolation, and occasionally disorganization of endoplasmic reticulum and Golgi complex at 48 hr. A single necrotic B-cell was identified in two of the islets from group VIII.

TABLE Mean value \pm S.E.M. of Blood Analyses of Groups of Starved Mice 10 min after Treatment. The Number of Observations is Given in Brackets

Group	Treatment	PI (mmol/l)	pH (pH-unit)	pCO ₂ (kPa)	Standard Bicarbonate (mmol/l)
I	Saline (controls)	2.56 \pm 0.08(9)	7.20 \pm 0.01(17)	6.58 \pm 0.1(17)	16.64 \pm 0.34(17)
II	D-Glucose	2.19 \pm 0.14(6)	7.27 \pm 0.02(6)	6.18 \pm 0.46(6)	18.33 \pm 0.99(6)
III	D-Mannose	2.03 \pm 0.10(6)	7.25 \pm 0.03(6)	6.30 \pm 0.49(6)	18.20 \pm 1.11(6)
IV	D-Fructose	1.53 \pm 0.10(9)	7.26 \pm 0.02(6)	5.93 \pm 0.78(6)	16.00 \pm 0.58(6)
V	D-Galactose	2.68 \pm 0.13(12)	7.21 \pm 0.02(7)	5.97 \pm 0.20(7)	16.16 \pm 0.69(7)
VI	Sodium lactate	1.93 \pm 0.21(6)	7.40 \pm 0.03(6)	6.88 \pm 0.42(6)	29.92 \pm 2.01(6)
VII	NaHCO ₃	1.45 \pm 0.17(6)	7.46 \pm 0.04(6)	6.61 \pm 0.66(6)	31.33 \pm 2.16(6)
VIII	L-leucine	2.12 \pm 0.06(9)	7.27 \pm 0.01(9)	5.81 \pm 0.27(9)	17.45 \pm 0.45(9)
IX	L-tyrosine	2.10 \pm 0.09(7)	7.21 \pm 0.00(6)	5.95 \pm 0.72(6)	18.50 \pm 0.86(6)
X	NaH ₂ PO ₄	4.50 \pm 0.48(6)	7.19 \pm 0.01(9)	6.68 \pm 0.23(9)	17.44 \pm 0.44(9)

Significantly different from controls at level of $p < 0.05$

ii Significantly different from controls at level of $p < 0.01$

c Significantly different from controls at level of $p < 0.005$

d Significantly different from controls at level of $p < 0.001$

preceding work (Boquist 1978) was found to decrease the B-cell sensitivity to alloxan.

MATERIAL AND METHODS

Animals, Treatment and Blood Sampling

The animals were male non-diabetic adult C57BL/6J +/+ mice from a local stock, kept under standard laboratory conditions at a constant temperature of 22°C. They had free access to water and a standard laboratory ration containing 1.0% calcium and 0.75% phosphorus. The contents of vitamin D₃ were approximately 600 IU per 100 g diet. The animals were deprived of food for 24 hr before the first injection and until after the blood sampling at the 4 hr observation time. The substances used for treatment were injected intraperitoneally under ether anaesthesia.

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Serum Pi, pH, pCO₂ and Standard Bicarbonate

The results of the laboratory analyses are given in Table 2. The serum concentration of Pi in the controls was 2.36 ± 0.08 mmol/l (mean \pm S.E.M.). In comparison with this there was a decreased level of Pi in the groups treated with D-glucose, D-mannose, D-fructose, sodium lactate, NaHCO₃, L-leucine and L-arginine. Serum Pi was not significantly altered in the group given D-galactose, but was increased in the group treated with NaH₂PO₄.

The hydrogen ion concentration was slightly increased in the groups treated with D-glucose, D-mannose, D-fructose and L-leucine, and markedly elevated in those given sodium lactate and NaHCO₃ but not significantly altered in those treated with D-galactose, L-arginine or NaH₂PO₄. Increased standard bicarbonate was observed in the groups given sodium lactate and NaHCO₃. The standard bicarbonate was not affected in the other experiments.

TABLE 1 Blood Glucose Concentration (mg/100 ml) in Starved Mice before (0 hr) Pre-treatment and at Different intervals after Treatment with Alioxan or Saline Injected 10 min after Pre-treatment. The Number of Observations Is Given in Brackets

		Blood glucose concentration (Mean \pm S.E.M.)																				
Pre-treatment	Treatment	Hours								Days												
		0		1		2		3		4		5		6		7						
Na	Saline	55.2	7	51.6	71	5	5	61	49	3	71	10	4	6	11	1.0	94.0	161	1	10	11	24.0
L-leucine	Alioxan	46.4	4 (61)	49.2	5 (81)	51	70.0	41.3	2	61	61.9	6	14	10.9	61	10	5	61	10	11	10.0	
L-arginine	Alioxan	56	5 (71)	149.0	0	71	5	67.0	67.0	5	0	71	7	71	2	5	54.0	71	11	2	71	
NaH ₂ PO ₄	Alioxan	54.4	6 (71)	76	14 (71)	60	71	5	5	71	1	71	71	11	11	13.1	1	71	2	71	71	
Na	Alioxan	5	5 (71)	165.1	12 (61)	12	91.0	49.5	21	9	0.5 (61)	71	1	16	1.12	11	71	17	1	3.7	71	

The animals were fed again after blood sampling at 4 hr. This explains the blood glucose elevation at 1 day in the controls and in the groups which were insensitive to aliolan, and might have contributed to the hyperglycaemia in the other groups.

groups. No significant difference was found between the groups in pCO₂ values.

Light and Electron Microscopy

Light microscopy of the pancreas disclosed typical B-cell islets (Boquist 1977; Boquist *et al.* 1977) 48 hr following aliolan injection in non-pre-treated mice (group XII), and in mice pre-treated with L-arginine (group IX) or NaH₂PO₄ (group X). Islets composed of unaffected B-cells, on the other hand, were encountered in mice treated with L-leucine (group VIII). A few B-cells with slight

cytoplasmic degeneration could be observed in a few islets from these mice.

Electron microscopy showed typical B-cell islets (Boquist 1977; Boquist *et al.* 1977) at both observation times in groups IX, X and XI (Fig. 1). The islets from group VIII were either normal or had occasional B-cells with mitochondrial lesions at 4 hr and a few B-cells with mitochondrial lesions, cytoplasmic vacuolation, and occasionally disorganization of endoplasmic reticulum and Golgi complex at 48 hr. A single necrotic B-cell was identified in two of the islets from group VIII.

TABLE 2 Mean \pm S.E.M. of Blood Analyses of Groups of Starved Mice 10 min after Treatment. The Number of Observations Is Given in Brackets

Group	Treatment	pH (mmol/l)	pH (pH-units)	pCO ₂ (kPa)	Standard Bicarbonate (mmol/l)
I	Saline (controls)	2.56 \pm 0.08(9)	7.70 \pm 0.01(17)	6.58 \pm 0.21(17)	16.64 \pm 0.34(17)
II	D-Glucose	2.19 \pm 0.14(6)	7.27 \pm 0.02(6)	6.18 \pm 0.46(6)	18.33 \pm 0.99(6)
III	D-Alucose	2.03 \pm 0.10(4)	7.25 \pm 0.03(4)	6.30 \pm 0.49(6)	18.20 \pm 1.11(6)
IV	D-Fructose	1.53 \pm 0.10(9)	7.26 \pm 0.02(6)	5.93 \pm 0.74(6)	16.00 \pm 0.58(6)
V	D-Galactose	2.68 \pm 0.13(12)	7.21 \pm 0.02(7)	5.97 \pm 0.70(7)	16.16 \pm 0.69(7)
VI	Sodium lactate	1.93 \pm 0.21(6)	7.40 \pm 0.03(6)	6.88 \pm 0.42(6)	29.92 \pm 2.01(6)
VII	NaHCO ₃	1.45 \pm 0.12(6)	7.46 \pm 0.04(6)	6.61 \pm 0.64(6)	31.33 \pm 2.16(6)
VIII	L-leucine	2.12 \pm 0.06(9)	7.77 \pm 0.01(9)	5.81 \pm 0.27(9)	17.45 \pm 0.45(9)
IX	L-arginine	2.10 \pm 0.04(7)	7.71 \pm 0.00(6)	5.95 \pm 0.72(6)	18.50 \pm 0.86(6)
X	NaH ₂ PO ₄	4.50 \pm 0.48(6)	7.19 \pm 0.01(9)	6.68 \pm 0.23(9)	17.44 \pm 0.44(9)

Significantly different from controls at level of $p < 0.05$

b Significantly different from controls at level of $p < 0.01$

c Significantly different from controls at level of $p < 0.005$

d Significantly different from controls at level of $p < 0.001$



Fig. 1. Electron micrograph of a portion of a pancreatic islet 4 hr after alloxan injection in group IX, demonstrating two degenerating (B) and two rather well-preserved (A) B-cells, one A (A) and one D- (D) cell without progressive alterations. The secretory granules are served in the degenerating B-cells but many are absent and exhibit atypical cores. The mitochondrion of A and D-cells are medium-sized. A prominent rough leoplasmic reticulum is observed in the A-cell, possibly consequence of the hypoglycaemia known to occur at the observation time following alloxan. $\times 8,000$

DISCUSSION

Alterations in blood glucose concentration and islet morphology formed the basis for estimation of B-cell sensitivity to alloxan. The B-cells are known to be particularly active in accumulating injected insulin (Hammarström *et al.* 1967). The criteria used for alloxan sensitivity were occurrence of a typical triphasic blood glucose response and selective B-cell lesions, whereas the criteria used for protection against alloxan were absence of triphasic blood glucose response and presence of islets which either were exclusively composed of normal B-cells or were composed of unaffected B-cells and only a few B-cells with slight or moderate degenerative changes.

The alloxan-antagonizing action of D-glucose is well known (See & Bhattacharya 1952; Bhattacharya 1954; Vitar Palesu *et al.* 1957; Carter & Smetana 1962; Kanelo & Lagorhopoulos 1963; Chazotte & Tadjel 1971; Zerkowich & Bender 1973; Rossini *et al.* 1974; Tomita & Kobayashi 1976), and a similar action of D-mannose (Bhattacharya 1954; Scherzias & Tadjel 1971) and D-fructose (Bhattacharya 1954) has been reported. In preceding studies in this laboratory a protection against alloxan toxicity was found in mice pre-treated with D-glucose, D-mannose, D-fructose (Boquist 1977) and (reported for the first time) also in mice pre-treated with sodium lactate and NaHCO_3 (Boquist 1977). In the present study a alloxan-antagonizing action was observed in mice pre-treated with L-leucine. No blood glucose alterations or any structural B-cell lesions were observed in mice pre-treated with this amino acid. The finding of a protective action against alloxan by pre-treatment with L-leucine contrasts with the report of Scherzias & Tadjel (1971) in their study of ob/ob-mice. These authors used other intervals between alloxan and test compounds, determined blood glucose only at 48 hr and did not study islet morphology. The use of L-arginine has not previously been reported in studies of alloxan sensitivity. In the present study pre-treatment with L-arginine did not decrease the B-cell sensitivity to

alloxan. The different effects of L-arginine and L-leucine on alloxan sensitivity are not completely unexpected in view of the well-known fact that these amino acids differ somewhat in their effects on the islets.

The protection against alloxan by sodium lactate and NaHCO_3 was in a preceding study found to be associated with metabolic alkalosis (Boquist 1978). The induction of metabolic alkalosis by treatment of mice with sodium lactate and NaHCO_3 was verified in the present study. In addition, a slight but significant increase in blood pH was observed in animals treated with D-glucose, D-mannose, D-fructose and L-leucine, but not in those given D-galactose, L-arginine and NaH_2PO_4 . The interval used between treatment and blood sampling for acid-base and Pi analyses, as well as the doses of the injected compounds, was the same as used in the alloxan sensitivity experiments. Thus, pH elevation was seen in mice treated with those compounds which in the present and/or preceding studies were found to protect against alloxan, but not in animals given those compounds which did not decrease the alloxan sensitivity. These findings support the opinion (Boquist 1978) that alkalosis, directly or indirectly protects against alloxan B-cell toxicity *in vivo*. Since it is known that acidity increases, and alkalinity decreases alloxan stability *in vitro* (Seligson & Seligson 1951), one possible explanation of the protective influence of alkalosis may be accelerated degradation of alloxan *in vivo*.

Serum Pi was significantly decreased after treatment with D-glucose, D-mannose, D-fructose, sodium lactate, NaHCO_3 , L-leucine and L-arginine, and, as expected, significantly increased in animals given NaH_2PO_4 . Unaffected serum Pi was observed in mice treated with D-galactose. Thus, with the exception of L-arginine, induction of hypophosphatemia was found after treatment with those compounds which protect against alloxan toxicity but not after injection of those compounds which do not decrease the alloxan sensitivity. Judging by these results and findings in parallel studies, it is believed that the concentration of Pi also plays a role in the development of alloxan diabetes. Studies are in progress on other aspects of this point.

One possible explanation for the decrease in serum Pi in the present study is glycolysis in peripheral tissues induced by insulin secretion. This may be the reason why hypophosphatemia was found also after administration of L-arginine. However the serum concentration of Pi is not affected in this kind of mice 10 minutes after intraperitoneal injection of insulin (unpublished observations). Therefore other factors may play a role in the development of the alterations observed

in serum Pi concentration, as well as in acid base balance.

The following working hypothesis is presented for subsequent studies. The inorganic phosphate and hydrogen ion concentration, extracellularly and intracellularly in the B-cells, is of importance in the development of alloxan diabetes (»Pi pH hypotesis«)

Supported by grants from the Swedish Medical Research Council Project No B 78 12X-00718 13A.

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POST STREPTOCOCCAL GLOMERULONEPHRITIS

A Quantitative Glomerular Investigation

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Ludwigsen, E. & Hanberg Sørensen, F. Post-streptococcal glomerulonephritis: a quantitative glomerular investigation. *Acta path. microbiol. scand. Sect. A*, 86: 319-324, 1978.

A light-microscopical, quantitative glomerular examination was performed on renal biopsies from 14 patients with acute post-streptococcal glomerulonephritis. A significant increase of 58% ($p < 0.0025$) in the total number of glomerular cells was found which was mainly due to an increase of endothelial cells (97%). Mesangial cells were, however, also increased (61%) and mesangial area was increased by 34% ($p < 0.05$). Biopsies from patients with persisting proteinuria and/or haematuria revealed slight hypercellularity and an increase in mesangial area during the first six months of disease. The abnormalities in the acute phase of the disease could be so slight that they only would be detected by a quantitative investigation.

Key words: Post-streptococcal glomerulonephritis, renal biopsy, differential cell count, morphometry.

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Accepted as submitted 28.8.78

Acute complex-mediated glomerulonephritis elicited by streptococci is one of the best known types of glomerulonephritis from a pathogenetical point of view (Gerrnack & Pollack 1958; McCracken *et al.* 1960). Morphologically the light-microscopic picture is dominated by an increase in the number of glomerular cells. This phenomenon, known during the prebiopsy period, was confirmed by the results obtained from biopsies (Jennings & Earle 1961).

Our knowledge of glomerular lesions in this disease (as in other glomerular diseases) is, however, nearly entirely derived from subjective judgments. Quantitative studies of biopsy material have been very few (Jadava 1968; Hefner 1974) and partly inconsistent as to the cell type mainly responsible for the tuft hypercellularity. Precise knowledge of the character and time-dependence of the glomerular lesions may have importance for classification and prognosis. Recent investigations of technical and statistical problems involved in

quantitative glomerular research (Hanberg Sørensen & Leder 1972; Hanberg Sørensen 1977) have set the stage for a study of glomerular disease. In this study an attempt is made to fill some of the gaps in our knowledge of the glomerular parameters of post-streptococcal glomerulonephritis.

MATERIAL AND METHODS

Criteria for Selection of Specimens

Percutaneous renal biopsies from patients with acute glomerulonephritis having an elevated titer of antistreptolysin O (ASO) and/or anti-hydroxase (AH) were selected from our files for quantitative investigation if they fulfilled the technical criteria of having at least ten undamaged glomeruli not situated near the outer surface of the biopsy specimen, and if it was possible to study 30 serial sections of each of these glomeruli to be sure that only central sections were used for the quantitative investigation (Hanberg Sørensen & Leder 1972; Hanberg Sørensen 1977).

The Patients

One biopsy from each of 14 patients, six females and eight males fulfilled these criteria. The mean age was 32.0 years (range: 7-76 years). Five patients had renal symptoms for 2 weeks or less, five patients for 2-4 weeks, three patients for 6 weeks, 8 weeks and 6 months, respectively and one patient had renal symptoms for several years. Ten patients had impaired renal function judged by serum-creatinine and/or creatinine clearance. Four patients had transient oliguria or anuria, and only these patients demonstrated a severe decrease in renal function. All patients had proteinuria. Thirteen patients had macro- or microscopic hematuria and two patients had had previous episodes of hematuria. A pharyngeal swab was performed in four patients, of which three showed group A hemolytic streptococci and one patient had a normal pharyngeal bacterial flora. Nine patients had elevated blood pressure of these four patients had a marked elevation (blood pressure $\geq 180/100$).

Six patients recovered within six months, two patients within 12 and 21 months. Two patients had persisting proteinuria and two patients a persisting, but slight elevation of blood pressure. Two patients relapsed within three weeks and six months, respectively after the initial attack. One recovered, the other had persistently elevated blood pressure. One patient, who died two years later of gastric cancer had had diabetes mellitus for 23 years. At autopsy her kidneys showed lobular glomerulonephritis. One patient had cerebral seizures and therefore transiently was given Decadronfosfat®. One received Endoxan® which was stopped because of pneumonia and one patient was transiently treated with Imurel®.

The Biopsy Specimens

A proliferative glomerulonephritis of varying degree was noted in almost all cases on conventional light microscopy but none had epithelial crescents. Immunofluorescence investigation was performed on twelve biopsies and in eleven deposits of IgG and/or C3 were found. In three biopsies IgA was also found. One biopsy showed no deposits one week after the start of the disease and one biopsy contained no glomeruli. Four biopsies were investigated by electron microscopy and humps were found in two one of which contained dense mesangial deposits also. Such deposits were present in a third biopsy. One specimen was without significant ultrastructural lesions one week after the start of the disease.

Methods

The quantitative examination of the biopsies was carried out as a blind study. The specimens were investigated together with renal biopsies from other renal diseases, the quantitative examiner (E.L.) being unaware of clinical data and histological diagnosis.

The biopsy specimens were fixed in Carnoy's fluid (six patients) or 4% aqueous formaldehyde (pH 7) (eight patients). Thirty serial sections of each paraffin embedded biopsy were cut on a microtome adjusted to 2 μ m and stained with periodic-acid Schiff hematoxylin.

The quantitative examination was carried out on central sections of ten different glomeruli (Hakky Sørensen & Leder 1972). If more than ten glomeruli were available, three glomeruli situated most subcapsularly, three most juxta-medullary and four glomeruli found in the mid-cortical zone were examined.

The quantitative examination included total and differential count of nuclei in all biopsies. Determination of mesangial and total glomerular areas (i.e. area delineated by Bowman's capsule) was carried out on biopsies fixed in Carnoy's fluid, because these parameters are dependent on fixation and the normal values used for comparison was fixed in Carnoy's fluid (Hanberg Sørensen 1977). The quantitative methods used in this study have previously been described in detail (Hanberg Sørensen & Leder 1972, Hanberg Sørensen 1977). Point counting was used for determination of total glomerular and mesangial areas, the point spacing being 17 μ m. The total and differential counts were carried out on projected glomeruli with marking of cell nuclei counted. A Leitz Ortholux microscope was used with a mirror for projection. The objective was a Leitz PL/Apo 40/075. Total magnification of the projected glomerulus was $\times 920$.

Statistical Methods

Estimation of means and the variances of means was performed as described previously (Hanberg Sørensen 1977). Comparison of the parameter means of biopsies with post streptococcal glomerulonephritis and normal was done by a modified t-test (Hald 1957).

RESULTS

In Table 1 the mean values of total and differential counts as well as total and mesangial areas are compared with normal values for these parameters provided by a study of renal biopsies from persons without renal disease investigated by the same technique as in the present study (Hanberg Sørensen 1977). On all 14 biopsies differential counts of nuclei were performed. On six Carnoy-fixed biopsies determination of areas was also performed. Of the 14 patients, two patients were excluded from the statistic estimations because of age (7 and 8 years old) because renal biopsies from children were not included in the normal material. One patient was omitted, because she had had diabetes mellitus for 23 years and another because he had had proteinuria for several years.

As shown in Table 1 there is a significant increase of 58% in the total number of cells. This increase is due to a proliferation of endothelial cells and mesangial cells which were increased by 97% and 61% respectively. The significant decrease in the percentage of epithelial cells is due to an increase in the number of endothelial cells. Mesangial cell per cent of total cells was not altered from normal

TABLE 1 *Quantitative Glomerular Findings in Post-streptococcal Glomerulonephritis (PSG)*

Parameter	Number of kidneys		Mean values and variances of means				Level of significance of difference
	PSG	Normals	PSG		Normals		
			\bar{X}	S^2	\bar{X}	S^2	
number of mesangial nuclei Age < 45 Years	10	10	40.3	36.51	25.0	0.77	$p < 0.0125$
number of endothelial nuclei All ages	10	22	94.0	207.93	47.7	2.86	$p < 0.0025$
number of epithelial nuclei Age < 45 years	10	10	39.3	8.22	34.1	1.45	
mesangial nuclei % of total nuclei Age < 45 years	10	10	23.0	4.56	23.7	0.66	
endothelial nuclei % of total nuclei All ages	10	22	52.7	8.52	43.2	0.36	$p < 0.0025$
epithelial nuclei % of total nuclei Age < 45 years	10	10	24.3	6.13	32.6	0.18	$p < 0.0025$
total glomerular nuclei All ages	10	22	173.4	351.16	110.0	14.70	$p < 0.0025$
total glomerular area mm^2 1,000 All ages	4	12	21.9	6.49	18.3	1.04	
mesangial area % of total area Age < 45 years	4	6	11.5	2.46	8.6	0.03	$p < 0.05$
total nuclei per 1,000 mm^2 of total area All ages	4	12	7.1	1.15	6.3	0.03	
mesangial nuclei per 1,000 mm^2 of mesangial area Age < 45 years	4	6	15.5	2.05	16.6	0.52	

variance values indicated considerable scattering in endothelial cell counts and thus of total cell counts also. Compared with normal values the mesangial area as percentage of total glomerular area was increased by 34% and the total glomerular area by a insignificant increase of 20%. The total cell number and mesangial cell number per area unit were the same as in normals.

In Fig. 1 the quantitative glomerular changes of each biopsy are presented in a diagram designed for individual biopsies (Hansberg-Sørensen 1977), which demonstrates the same trends as described above. It also appears that some of the patients have nuclear counts and an endothelial number of nuclei expressed as percentage of the total number of

nuclei which are within the 95% confidence limits.

In order to assess the effect of length of disease the data of each biopsy was related to duration of renal symptoms. Fig. 2 shows the fluctuation of the total cell count with time. A peak within the first two weeks of disease is seen but hypercellularity is still present nearly six weeks after the onset. Hereafter values are just above or in the upper range of normal values. Of the two patients who had counts within normal limits after 25 and 28 days one showed a significant increase in total glomerular area and a number of endothelial nuclei as percentage of total number of nuclei in the upper normal range. An area determination was not performed on the biopsy from the other patient, but

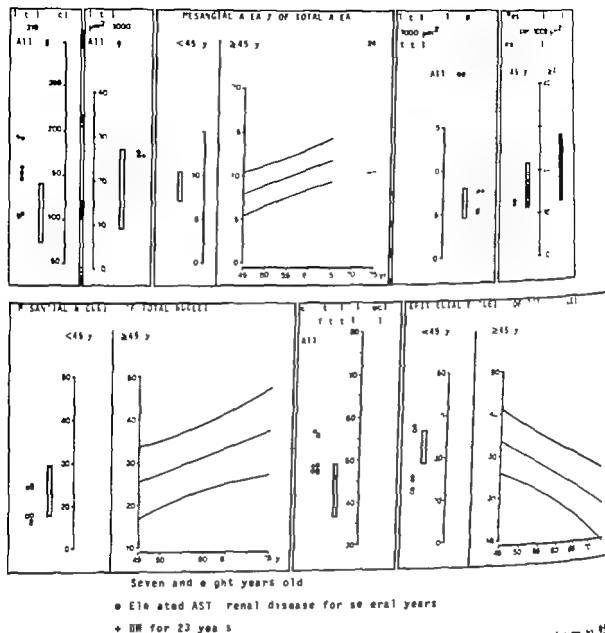


Fig 1 Mean values in biopsies from patients with post-streptococcal glomerulonephritis in relation to 95% confidence limits for normal renal biopsies

the differential nuclei count showed a significantly increased number of endothelial nuclei as percentage of total number of nuclei. Both had significantly elevated AH and had deposits of immunoglobulin and C3. In addition one had many humps and dense deposits in mesangial location on electron microscopy. The hypercellularity was due mainly to a proliferation of endothelial cells, the values of which were continuously significantly elevated (Fig. 3).

After two months one patient had a significantly elevated relative mesangial area (mesangial area per cent of total area was 15.9% normal range being 6.9–10.3%). The mesangial cell number per unit area for this patient was in the upper normal range.

DISCUSSION

Some of our results are in accordance with those of Wehner (1974) the only investigator to have performed a quantitative glomerular investigation on a larger number of patients with acute glomerulonephritis. He found in ten patients with slight and ten patients with moderate proliferative glomerulonephritis an increase in the total number of cells by 50% and 80% respectively. Contrary to this however he found the increase to be mainly due to a proliferation of mesangial cells. It is possible that difficulty in distinguishing between endothelial and mesangial cells, if these are not situated in very typical location may explain this divergence.

number of nuclei

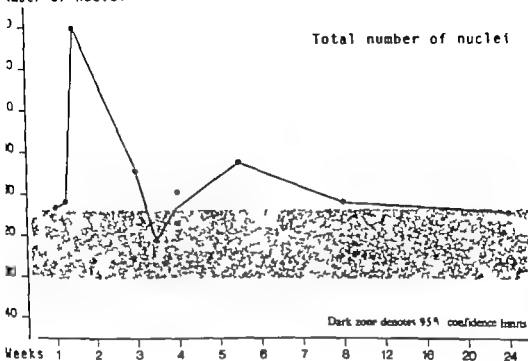


Fig 2 Fluctuation of total number of nuclei in relation to duration of disease

number of nuclei

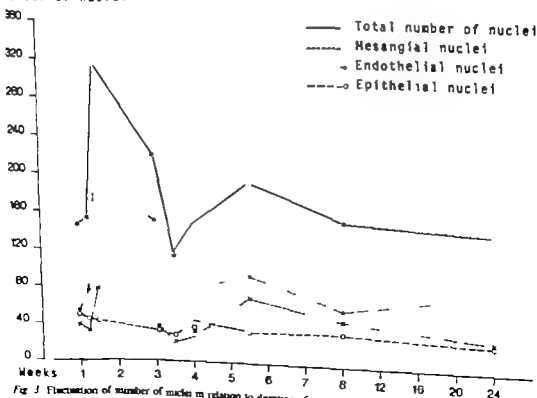


Fig 3 Fluctuation of number of nuclei in relation to duration of renal disease

Another explanation might be that the duration of the disease was different in the two series. The paper of *Wehner* contained no information on the time lapse between onset of the disease and the biopsy. It seems (Fig. 2) that endothelial proliferation is characteristic for the early phase of the disease, a feature also known from subjective experience (*Jennings & Earle* 1961). In later phases (and following clinical recovery) mesangial hypercellularity may be most prominent as demonstrated by quantitative methods on an autopsy material (*Hara* 1972). Epithelial cells did not contribute to glomerular hypercellularity in our biopsies as noted by others (*Wehner* 1974, *Iidaka* 1968 and *Hara* 1972).

Our demonstration of an increase in mesangial area is in accordance with the quantitative studies by *Iidaka* 1968 and *Hara* 1972 but not with the results of *Wehner*. This might be due to a difference in technique, *Wehner* using silver-stained specimens and also quantitating on peripherally situated sections while we quantitate on specimens stained with periodic acid Schiff hematoxylin and only use central sections.

One result of our study is that some cases of acute post-streptococcal glomerulonephritis studied early (i.e. during the first three-four weeks) did not show an increased number of total cells or had such slightly elevated cell counts that the glomeruli would be judged as normal by routine subjective judgement. This was also seen in cases in which immunodeposits could be demonstrated. The significance of this for general diagnostic purposes is that a normal or «minor change» biopsy is compatible with acute complex nephritis. This stresses once more the importance of the routine combining of light microscopy with immunofluorescence and electron microscopy in renal biopsy diagnosis.

We wish to thank *Claus Bruun*, M.D. Department of Clinical Pathology, Municipal Hospital, Copenhagen for use of his material and for six biopsies. For providing us with the results of immunofluorescence investigation we wish to thank *Svend Larsen*, M.D. Department of Clinical Pathology, Municipal Hospital, Copenhagen and *Erik Sommer Hansen*, M.D. University Institute of Pathology, Kommunehospitalet, Aarhus.

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PERIODIC ACID SCHIFF POSITIVE NON-GLYCOGENIC GLOBULES IN HEPATOCYTES

Differential Diagnostic Aspects in Screening for Alpha 1 Antitrypsin Globules in an Autopsy Material

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Reintoft, I. Periodic Acid Schiff-positive non-glycogenic globules in hepatocytes. Differential diagnostic aspects in screening for alpha 1-antitrypsin globules in an autopsy material. Acta path. microbiol. scand. Sect. A 86: 325-329, 1978.

Thirty-eight subjects with disease-resistant PAS-positive cytoplasmic globules in hepatocytes were found among 238 autopsies. In 15 of the 38 subjects the globules were antiproteolytically alpha-1-antitrypsin, in 23 subjects they were not. The latter globules were found in centrilobular regions, the alpha-1-antitrypsin globules mainly in periportal regions. The non-alpha-1-antitrypsin globules showed less differences in size (6-10 μ) and a smaller number per hepatocyte (1-7) than the alpha-1-antitrypsin globules (1-40 μ and 1-30 per cell). The non-alpha-1-antitrypsin globules were only demonstrated in livers with centrilobular sinusoidal dilatation having, in all cases but one, also centrilobular confluent necrosis. This type of globules can be assumed to be of differential diagnostic importance mainly in an autopsy material. The nature of these globules is discussed.

Key words: Liver, alpha-1-antitrypsin globules, PAS non-glycogenic globules.

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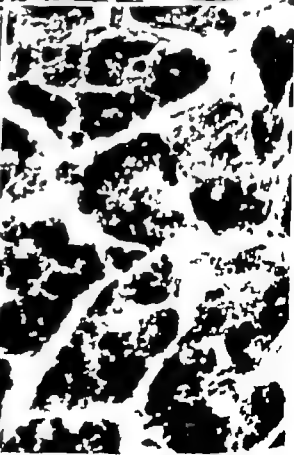
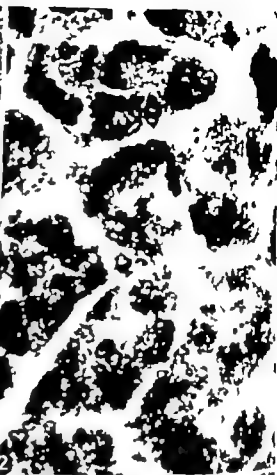
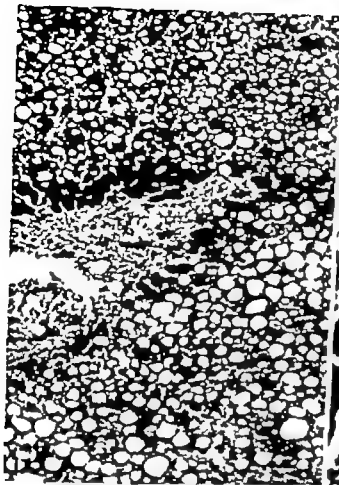
Received 15 vii 77 Accepted 3 vii 78

Alpha-1-antitrypsin (A 1 AT) is formed by the hepatocytes. Persons having alpha 1-antitrypsin deficiency (A 1-ATD) of genotype Z form an antitrypsin which is difficult to dissolve and apt to agglutinate. It accumulates in the endoplasmic reticulum of hepatocytes, where it is demonstrable as diastase resistant periodic acid Schiff (PAS)-positive globules (3, 9). Such globules have been found with considerable constancy and they seem to be the histological evidence of the Z gene (1, 6, 11, 19).

Preliminary results in autopsies have shown that some diastase-digested, PAS-positive globules give a positive immunoperoxidase reaction, others negative. The purpose of this investigation was to investigate other possible histological and histochemical differences.

MATERIAL AND METHODS

The material comprises liver tissue from 238 autopsies. The method of collecting the liver tissue has been described previously (18). All liver tissue was fixed for 24 hours in cold formalin at pH 7.4, embedded in paraffin, and cut into 2 μ thick sections. The sections were screened for cytoplasmic globules in the hepatocytes after diastase digestion and PAS staining. A magnification of $\times 250$ was used. Positive globules - which were round, homogeneous, well demarcated, and greater than 1 μ - were recorded, whereas small irregular globules and granules such as lipofuscin were not. The sections with positive globules were also stained with haematoxylin-eosin (H.E.), Masson trichrome connective-tissue, long Ziehl-Neelsen acid fast, Mallory phosphotungstic acid haematoxylin (PTAH) (12), alcian blue of pH 2.6 with van Gieson (alcian-Eckford) staining (5), Perls' method for iron, alkaline Congo red method of Puchtler, Sweat & Levine (17), Gordon & Sweet



nodes staining (7), and the immunoperoxidase reaction demonstrate antigenic identity of the globules with A1 AT. For the immunoperoxidase reaction the 3-layer immunostyrene bridge technique of Palmer et al (13) is used with the modifications described previously (3).

Immunoperoxidase-stained preparations were compared with PAS-stained ones to assess the localization of α globules. In addition, the immunoperoxidase-stained preparations were later PAS stained, omitting diastase digestion.

The following morphological findings were recorded: without, centrilobular sinusoidal dilatation (16), and centrilobular confluent necrosis (20).

RESULTS

Diastase-resistant, PAS-positive globules were found in the specimens from 38 subjects. Fifteen of these (group I) gave a positive A1 AT staining which showed an annular darkbrown reaction with less intense central staining or diffuse, granular staining (Figs. 1-2). Twenty-three subjects (group II) were negative in A1 AT staining.

Group I: Globules were found in liver cells adjacent to portal spaces (Fig. 3) or in the vicinity of fibrous septa. In many sections they were present only in small and scattered groups of liver cells. However the patient with liver cirrhosis had numerous globule-containing hepatocytes. Each cell might contain from 1-30 globules, as a rule measuring 1-10 μ , but a few 20-40 μ . The globules stained deeply with PAS-following diastase digestion and had sharp limits.

Comparison of immunoperoxidase- and diastase PAS-stained sections revealed that PAS-positive globules were present in the same zones that were positive in the immunoperoxidase reaction. In the combined immunoperoxidase and PAS-stained sections (Fig. 4) there were PAS-positive areas corresponding to the centre of the annular immuno-

peroxidase-positive areas, while no PAS-positivity could be demonstrated in the diffuse granular immunoperoxidase-positive areas.

The liver tissue in the 15 group I cases showed in 11 cases centrilobular sinusoidal dilatation plus centrilobular liver cell necrosis in 8 of them. 2 cases were without dilatation, one case was unassessable because of pronounced infiltration by tumour and one had cirrhosis. Five of the fifteen group I cases had globules as in group II in addition to the A1 AT globules. All 5 cases showed sinusoidal dilatation, 4 with centrilobular necrosis. Thus of the 11 cases with centrilobular sinusoidal dilatation 5 cases contained globules of group II.

Group II: Globules negative in A1 AT staining occurred most often in the hepatocytes centrally in the lobules (Fig. 5) or peripherally to central necroses.

In general, the number of globules in group II were much fewer than in group I, and an average of 1-7 globules were found in each affected cell. They were of equal size, measuring 6-10 μ . The globules did not stain as reddish-blue by PAS as the globules

Fig. 1 Survey showing immunoperoxidase-positive areas around the portal spaces. \times approx. 130.

Fig. 2 Anisotropy reaction with less intense central staining as well as diffuse granular intracytoplasmic staining. Immunoperoxidase reaction. \times approx. 900.

Fig. 3 At the top part of a portal space with numerous cytoplasmic globules in adjoining hepatocytes. PAS following diastase digestion, no nuclear staining. \times approx. 450.

Fig. 4 Same section as in Fig. 2, after PAS staining. Now the circles are filled, and some of the granules appear somewhat coarser. Immunoperoxidase reaction and PAS staining. \times approx. 900.



Fig. 5 Top right: Central vein with numerous cytoplasmic globules in adjoining hepatocytes. PAS following diastase digestion, no nuclear staining. \times approx. 450.

TABLE 1 Number of Cases Showing Histochemically Stainable Globules

	Number of cases Group I	Number of cases Group II
Diastase-resist.		
PAS-pos.	15	23
Immunoperoxidase	15	0
Masson trichrome	10	11
Long Ziehl Neelsen	4	2
PTAH	3	2
Aldan Eskelund	2	6
H. E.	3	0
Perla	0	0
Alkaline Congo	0	0

in group I being more pinkish. The demarcation against the surrounding cytoplasm was not as distinct as in the case of group I globules.

In 22 cases the hepatic tissue exhibited centrilobular sinusoidal dilatation and centrilobular liver cell necrosis. One patient had cirrhosis.

The results of the histochemical studies in groups I and II are listed in Table 1. On Masson trichrome staining the large globules ($> 15 \mu$) seen in group I stained a uniform red, the medium-sized ones ($7-15 \mu$) red with a blue ring and the small ones ($< 7 \mu$) blue. Within group II only blue globules were seen. On long Ziehl Neelsen PTAH and alcian Eskelund staining the globules in the two groups gave identical staining reactions, reddish brown, black (in some cases surrounded by a pale tan zone) and orange red respectively. In 3 cases the periportal globules of group I could be demonstrated by H. E. staining as homogeneous eosinophilic bodies. Group I globules were found in the same zones as the immunoperoxidase positive globules. Group II globules could not be demonstrated by H. E. staining.

DISCUSSION

The morphology and staining properties of the group I globules in this work corresponds to the previously described globules in A I ATD (1, 2, 6, 11, 19). On H. E. staining only 3 cases exhibited eosinophilic accumulations at the site of the globules but such accumulations are known to be often inconspicuous enough to be overlooked on H. E. staining (6, 11). The diastase-resistant PAS-positive and immunoperoxidase-positive globules occur especially in the vicinity of the portal spaces and fibrous septa in A I ATD (1, 21), but in autopsy materials similar globules have been claimed to be found around the central veins (6, 11). This was not

observed in the present study. Only two cases, and exceptionally many A I AT globules showed globule-containing cells also in the centre of the lobules.

The non-A I AT non-glycogenic PAS-positive globules, the group II globules, are fewer per hepatocyte and are of more equal size than group I globules. The homogeneous globules do not stain quite as deeply reddish-blue in PAS, and the demarcation, though sharp, does not appear as distinct as in group I globules. In the present study the globules were found only in liver tissue from autopsies showing centrilobular sinusoidal dilatation and in all cases but one also centrilobular liver cell necrosis. Thus, the non-A I AT globules seem to bear a relation to congestion and anoxia. On the basis of the existing material nothing definite can be said about these relations, since approximately 1/3 of a total of 238 autopsy livers showed sinusoidal dilatation and of those only approximately 20% contained the non-A I AT globules. It was tried to semiquantitate the degree of centrilobular congested necrosis and correlate this to the existence and non-existence of the non-A I AT globules, but no definite relationship was found.

The non-A I AT non-glycogenic PAS-positive globules have previously been observed in an autopsy study in which screening for A I AT globules was performed (4, 8), and globules of this type are known from animal livers as well. Peyer *et al.* (15) have described various diastase-resistant PAS-positive intracytoplasmic droplets in human livers. Droplets up to 8μ in diameter are believed to be lysosomes imbued with serum protein which has entered the cell because of e.g. an increase in cell permeability due to anoxia. Similar bodies have been described by Latal Pfeifer and Bauwens (14) and by Kerr (10) in liver cells in cases of hypertrophy caused by partial hepatectomy or other liver damage. They arise when blood plasma forces its way into the hepatocytes (14). An increased intrasinusoidal pressure is assumed to play an important role in the pathogenesis of the inclusion (14). It is not clear from the literature whether non-A I AT non-glycogenic PAS-positive globules have been observed in human liver biopsies. The author has observed such globules in a percutaneous liver biopsy taken from a woman 7 hours before she died.

More than half the cases of diastase-resistant PAS-positive intracytoplasmic globules in the present autopsy material bore no relation to A I AT and the study confirms the opinion (21) that the antigenic identity of diastase-resistant, PAS-positive globules with A I AT must be proved either by the immunoperoxidase or by the immunofluorescent

technique. By other special staining methods, it was not possible to differentiate with certainty between nodules of the A1AT type and of other types.

The author is indebted to professor Hemming Poulsen, Department of Pathology Hvidovre Hospital, for useful discussions and helpful correction of the manuscript.

Thanks are due for technical assistance to Janet Heide Jørgensen.

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CORRESPONDENCE

ANTIGENICITY OF ^{90}Sr INDUCED TUMOURS

Received 25 xi 78

Sir - In a recent article *Larsson, Lorentzon and Bergqvist* (Acta path. microbiol. scand. Sect. A, 85: 433-466, 1977) set out to «examine further whether or not BCG treatment of the ^{90}Sr -treated mice influences the total tumour incidence, the time of tumour development, the growth rate and the histological character of the tumours». The authors present data which agree with investigations reported in 1965 by *Johnson et al* (1) in that BCG was found to suppress the growth rate of ^{90}Sr induced tumours but had no influence on the time of tumour development, survival time of the animals, or distribution of tumours in the skeleton. However, in contrast to the earlier report (1) *Larsson et al* did not register any decreased incidence of tumours after treatment with BCG.

Larsson et al laid great emphasis on their negative findings in view of what they consider «optimum stimulation of immunological reactivity» in administering BCG in 4 doses every 3 weeks from 70 to 135 days after ^{90}Sr was given. Since, in the particular mouse strain used, the great majority of tumours arise approximately 200 days after ^{90}Sr treatment and appear only in few exceptional cases after shorter survival (2), their BCG treatment was made in our opinion during a period which is indeed far too early to have an influence upon developing neoplasms. As a general rule, the osteoblastic cell elements and their precursors are - during this period and with the doses used - strongly suppressed and will not, except on rare occasions, start to proliferate until approximately 180 day after the administration of ^{90}Sr . The successful demonstration of BCG-inhibition of tumour incidence in our experiments may therefore be due to the fact that BCG treatment was given at a time closer to the expected microscopic appearance of tumours and it could be therefore more effective.

It is conceivable that the difference in the amount of ^{90}Sr given to the animals could be also of importance with regard to the difference in tumour incidence noted by *Larsson et al*. They treated the animals with 0.7 μCi per gram body weight (dose giving a dose less than administered in our investigations (0.9 μCi per gram body weight). It has been suggested (3 and 4) that the development of antigenic tumours can be facilitated in an organism in which immune reactions are depressed by radiation. Thus our animals may have developed more antigenic tumours than those of *Larsson et al* since our animals were exposed to larger radiation dose.

Finally *Larsson et al* suggest that tumour-unrelated

deaths in the ^{90}Sr and BCG treated group might have introduced errors in the interpretation of our data. As *Larsson et al* point out correctly, there are indeed 40.8 per cent deaths without tumours among our ^{90}Sr and BCG-treated experimental animals, and not more than 14.3 per cent among the ^{90}Sr -treated control animals. However, it is clearly indicated in our table to which *Larsson et al* referred (1), that the mean survival time of the animals does not differ significantly from each other in any of the groups. Therefore, the conclusion drawn from these data and questioned by *Larsson et al* will remain correct.

We feel that the above points should be clarified in order to avoid confusion which may arise in view of some contradictory interpretations.

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Sir - The correspondence poses many important facts which underline the difficulties of interpreting and comparing the immuno-stimulating effect of BCG in ^{90}Sr induced osteosarcomas obtained in the studies by *Nilsson et al* (1965) and by us (1977). As we have pointed out in the discussion of our paper, the best effect of the presumed immunostimulation would be obtained at the time of the very first histological appearance of tumour cells, i.e. before the expected gross appearance of tumours. According to *Johnson* (1962), CBA mice having a body weight of 25-30 grams and being approximately 75 days old at the time of the radioisotope injection, develop the first histologically detectable tumour at 90 days after a dose of 0.67 microcurie ^{90}Sr per gram of body weight, and intramedullary osteosarcoma body

from 120 to 180 days after the injection of ^{90}Sr . Therefore, we preferred to give the BCG treatment from 70 to 135 days after the administration of 0.7 microcurie ^{90}Sr per gram of body weight to CBA mice of corresponding body weight and age. The fourth injection of BCG we gave at 135 days after ^{90}Sr can therefore be regarded as an optimum stimulation of immunological reactivity towards the first appearing antigenic, potentially neoplastic cells. There is the possibility that a larger dose of ^{90}Sr which was used by Nilsson *et al* (1965), might induce tumours which are more antigenically active than those appearing after a lower dose.

As the correspondents correctly point out their table (Nilsson *et al* (1965)) clearly indicates that the mean survival time of the animals does not differ from each other in any of the groups. We do agree to the conclusions drawn from these data as far as the given observation period is concerned. However because of the forty per cent death rate not related to tumour in the group of animals given BCG treatment, a possible late occurrence of tumour could not be studied. The longer survival period for the animals in our study gave a total tumour-incidence of 89.5 and 90.5 per cent for the respective groups of animals.

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BRIEF REPORT

UROTHELIAL HYPERPLASIA OF THE URINARY BLADDER OF THE RAT INDUCED BY MECHANICAL PERFORATION AND PHENACETIN TREATMENT

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Johansson, S. Urothelial hyperplasia of the urinary bladder of the rat induced by mechanical perforation and phenacetin treatment. *Acta path. microbiol. scand. Sect. A*, #6 333-335 1978

Mechanical perforation of the urinary bladder of Sprague-Dawley rats and subsequent administration of phenacetin in the diet induced urothelial hyperplasia in 11 of 12 rats. No pathological changes were found in the bladders of the control rats only submitted to mechanical perforation or phenacetin treatment only. The hyperplastic changes varied from mild focal urothelial hyperplasia after one week to severe focal and diffuse nodular and papillary hyperplasia after 3 weeks.

Key words: Phenacetin, urothelial hyperplasia, mechanical perforation.

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Accepted as submitted 2 v 78

The association between a heavy intake of phenacetin-containing analgesics and the development of renal pelvic tumors is well established (Johansson *et al* 1974). Primary tumors of the urinary bladder in phenacetin abusers have also been reported (Johansson & Wahlqvist 1977).

Urothelial hyperplasia of the renal papillae has been induced in rats after long-term treatment with phenacetin (Johansson & Aron *et al* 1976). Hyperplastic changes were also found in a few control rats but there was a significant difference in the degree and severity between the phenacetin-fed rats and the control rats. The hyperplastic changes were associated with vascular changes and/or calcification. In the above study also 2 out of 30 rats developed mild urothelial hyperplasia of the bladder. In a study by Toyokawa & Layton (1975) trauma to the bladder wall, caused by insertion of chalk powder and subsequent formation of bladder calculi, induced papillomatous and hyperplasia of the bladder mucosa. In their experiment, which lasted for up to 6 weeks, no malignant tumors developed.

This paper is a brief report of a study concerning the combined effects of mechanical trauma and phenacetin on the urinary bladder of the rat.

Material and Methods

Experimental design. Twenty male Sprague-Dawley rats weighing approximately 100 grams, supplied by Altrex AB, Stockholm, were used. Four of the rats were put on a diet containing 0.535% phenacetin. Sixteen of the rats were anesthetized with phenobarbital, the abdominal cavity was opened and the urinary bladder performed twice at the dome with a 23 gauge needle. Twelve of these 16 rats were then put on a diet containing 0.535% phenacetin, and 4 rats were given ordinary rat pellets without phenacetin. Four of the perforated phenacetin-fed rats, one non-perforated phenacetin fed rat and one control rat were sacrificed after one and after 2 weeks. The remaining phenacetin-fed rats (4+2) and 2 control rats were sacrificed after 3 weeks. The rats were weighed and the food consumption determined every week throughout the experiment.

Morphological methods. The urinary bladder was inflated with Bouin's solution. The bladder was divided into 2 parts and embedded in methacrylate and paraffin. Five μ m sections from the paraffin block and one μ m sections from the methacrylate block were stained with hematoxylin and eosin and according to Weigert-van Gieson. Histological examination of the kidneys was also performed.



Fig. 1 Severe urothelial papillary hyperplasia of the urinary bladder of the rat after mechanical perforation and two weeks of phenacetin treatment. H&E $\times 120$

Results

Three out of the 4 «perforated» rats showed mild focal urothelial hyperplasia after one week of eating phenacetin. Eight rats had diffuse papillary and partly nodular hyperplasia after 2 and 3 weeks of eating phenacetin (Fig. 1). Urothelial hyperplasia was found in the perforated areas as well as in areas distant from the areas of perforation. In 2 of the rats the entire bladder mucosa was involved. All of the control rats as well as the «non-perforated» phenacetin-fed rats had a normal bladder mucosa. The renal pelvis and papillae of all rats were of normal appearance.

Comments

Mechanical perforation of the urinary bladder followed by administration of phenacetin in the diet induced urothelial hyperplasia in 11/12 rats after 1 to 3 weeks of feeding phenacetin. All of the control rats which were only subjected to mechanical perforation or «non-perforated» phenacetin fed rats had normal bladder mucosa. These findings indicate that ingestion of phenacetin in combination with mechanical trauma promotes proliferation of the urothelium of the bladder in Sprague Dawley rats. In another study Sprague Dawley rats were fed for up to 110 weeks the same dose of phenacetin in the diet as in the present study. Only 2 of 30 rats developed mild urothelial hyperplasia of the bladder one of which also had moderate cystitis (Ukeshagen & Aspervall 1976). In 26 of the 30 rats urothelial hyperplasia of the renal papillae was found often associated with vascular changes and/or calcification.

A concentration of N-hydroxylated phenacetin metabolites in the renal papillae of the rat after ingestion of phenacetin has been suggested (Aerr 1971). Therefore, the localization of the lesions in the renal pelvis and not of the bladder may be caused by a higher concentration of phenacetin or N-hydroxylated metabolites of phenace-

tin in the urothelium of the renal papillae. Also the vascular changes and calcification may provide a less *minoris resistentiae* and be of pathogenetic importance for the development of urothelial hyperplasia, equivalent to the trauma to the bladder wall in the present study. The carcinogenic N-hydroxy esters of aromatic amines and amides (phenacetin is an aromatic amide) are highly reactive and shortlived (Miller 1970) therefore a lesser amount of the active substance may be expected to reach the bladder.

It is not possible to determine whether the urothelial hyperplasia of the renal papillae after long term feeding of phenacetin or the similar changes induced in the bladder after mechanical trauma and phenacetin treatment are reactive or preneoplastic. The administration of the nitrofurantoin 4-(nitro-2-furyl)-2-thiazolyl formamide (FANFT) in the diet to rats induces epithelial lesions of the bladder. The lesions progress from mild focal hyperplasia after 2 weeks to macroscopic carcinomas after 25 weeks (Cohen et al 1976). If FANFT was discontinued after 6 and 10 weeks, respectively the hyperplastic changes reverted to normal in the former group and progressed into invasive carcinomas in the latter (Cohen et al 1976).

By scanning electron microscopy it has been demonstrated that after 8–10 weeks of feeding FANFT there is an appearance of pleomorphic microvilli on the surface of the hyperplastic lesions. These pleomorphic microvilli seemed to indicate irreversibility, i.e. the hyperplastic lesions will not revert to normal but progress into a tumor (Jacobs et al 1976).

Scanning electron microscopy may be used to determine whether the epithelial lesions induced by mechanical trauma in combination with phenacetin treatment are reactive or preneoplastic. Studies of discontinuation of phenacetin after different time-intervals of feeding phenacetin following mechanical trauma would also be necessary.

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AMELOBLASTOMA OF THE JAWS

An Analysis of a Consecutive Series of all Cases Reported to the Swedish Cancer Registry during 1958-1971

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Larsson, Å. & Almerén, H. Ameloblastomas of the jaws. An analysis of a consecutive series of all cases reported to the Swedish Cancer Registry during 1958-1971. *Acta path. microbiol. scand. Sect. A*, 86: 337-349, 1978.

All the 49 jaw ameloblastomas reported to the Swedish Cancer Registry during the period 1958-1971 were re-examined, using the histopathologic criteria of odontogenic tumors adopted by WHO. Thirty-one cases (63.3%) fulfilled the criteria of simple ameloblastomas, whereas 12 cases were found to be other benign lesions and 6 to be malignant tumors other than ameloblastomas. The relationship between simple ameloblastoma, malignant ameloblastoma and other epithelial jaw tumors is discussed.

Key words: Ameloblastoma, jaw, Swedish Cancer Registry.

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Accepted as submitted 6 II 78

Although a rare tumor the ameloblastoma of jaw-bones has still been the subject of more studies than any other odontogenic tumor (see reviews by Gorlin *et al.* 1961, Krawer 1963, Smith 1968). The reasons for this interest in the ameloblastoma are manifold, e.g. there is a lack of agreement among investigators not only about the cell of origin, but also about the classification, behaviour and treatment of the tumor.

The histopathologic criteria of ameloblastoma have been defined by Finkelberg *et al.* (1971). Barker (1965) found that more than 50 different names had been given to the ameloblastoma. All of these names are related to the histologic appearance of the tumor, such as pleomorphic, follicular, squamoid, basal-cell, hemangioid, cystic, mural and granular cell ameloblastoma. Other names, such as melanotic, adeno- and fibro-ameloblastoma, denote other and well-defined entities separate from ameloblastoma (Krawer 1963, Gorlin 1970). Similarly the craniofacial angiosarcoma and the so-called adamantinoma of long bones are commonly regarded as specific entities, which should be differentiated from jaw-bone ameloblastoma (Smith 1968).

There seems to be a general agreement in the literature that the different histologic patterns of ameloblastoma are without prognostic significance. Some of the authors go so far as to suggest that the elaborate classification of ameloblastoma should be excluded from the terminology in order to avoid further confusion regarding therapy planning (Krawer 1963, Smith 1968). On the other hand, some of the subtypes of ameloblastoma show similarities to other types of tumors, such as benign and even malignant salivary gland tumors as well as epidermoid carcinoma; this may cause diagnostic confusion and therapeutic errors. Furthermore, pathologists have tended to classify unknown oral tumors, and particularly odontogenic tumors, into types of ameloblastoma. There is also the question of malignancy in ameloblastoma, which apparently has not yet been adequately settled. From the differential diagnostic point of view a histologic subdivision of ameloblastoma may thus still be justified, and particularly so in studies of the incidence and frequency of ameloblastoma have to be based on material collected from large series of cases from different sources.

Waldron stated in 1966 that there was a lack of

well-documented long-term follow-up studies of large series of patients with ameloblastoma. If retrospective and in order to be valid such studies would require an access to primary surgical material. With a few exceptions (Masson *et al* 1959 Meerkotter 1969 Mehlish *et al* 1972 Schdev *et al* 1974) this has not usually been the case in studies on the incidence and frequency of ameloblastoma.

As to the incidence of ameloblastoma, i.e. the number of tumors in a defined period of time in a defined population, such figures are usually difficult to obtain, particularly regarding larger populations like whole countries. The occurrence of ameloblastoma is expected to be about 1-2.3% of the tumors and cysts in and around the maxilla and mandible (Small and Waldron 1955 Meerkotter 1969).

In order to study the incidence of ameloblastoma in Sweden and also to establish a basis for long-term follow up studies of treatment results of ameloblastomas the purpose of the present study was to re-examine and evaluate histologically all ameloblastomas reported to the Cancer Registry in Sweden during the period 1958-1971.

MATERIALS AND METHODS

All cases diagnosed as ameloblastoma or adamantinoma during the period 1958-1971 comprising a total of 49 patients, were collected from the Swedish Cancer Registry. This Registry started its activity in 1958 and its main functions are to provide data and statistics for the study of cancer disease in Sweden. Statistics have been published from the Cancer Registry every year between 1958 and 1971 in a series called "Cancer Incidence in Sweden". From the latter publications the total number of bone and skull-face tumors was also collected in the present study in order to determine the frequency of ameloblastoma relative to all registered bone tumors in Sweden.

It is compulsory for a Swedish physician and pathologist to report on all clearly malignant neoplasms, including also cases of certain types, irrespective of whether they are malignant or not, for example adamantinoma or ameloblastoma. In 95% of all cases registration is based on a double report, one from the clinician and one from the pathologist. The remaining 5% of cases are diagnosed only through a clinical examination, a radiogram, or at an operation. The identification of the patients is based on the national registration number. All data are fed to a computer including codes recording the patient's place of residence, occupation and the diagnosis of tumor. The coding system is based on the international classification of disease, and the histopathological diagnosis corresponds to that of the WHO list (WHO/HS/CANC 24.1 Histology Code).

The original diagnosis in all the cases of the present study had been made at histopathological examination of

primary biopsy or surgical material. On the basis of the computerized data received from the Cancer Registry the records and the specimens of all the 49 cases were requested from the clinics and the pathology departments. In addition to collecting all material available from the surgical operation, an effort was also made to obtain all preoperative biopsy material for re-evaluation. Sectioned and/or paraffin-embedded material was available in all the 49 cases. The following histological stains were used at re-examination: haematoxylin-eosin, van Gieson's stain and PAS. The application of statistics was initially considered but was found to be unnecessary. We intend to apply statistical treatment to selected data of the present material in a subsequent clinical re-examination.

RESULTS

Histopathological Examination (Table 1)

Re-examination was performed using the histopathologic criteria of odontogenic tumors adopted by WHO (Pindborg *et al* 1971).

1. *Cases re-confirmed as ameloblastoma.* Upon re-examination, thirty-one (31) of the 49 registered cases of ameloblastoma fulfilled the criteria of simple ameloblastoma (Table 1). Among these were cases of a predominantly follicular (Fig. 2), plexiform (Figs. 29-30) and acanthomatous type (Fig. 6), and also cases exhibiting a mixture of the three types. Three of the ameloblastomas were predominantly cystic in appearance, and six were considered to represent early ameloblastomas arising in the wall of a cyst (Figs. 11-12).

Malignancy had been primarily considered in six of the 31 cases that were found upon re-examination to be simple ameloblastoma (cases 4, 19, 25, 29, 39, 45). In three of these cases (25, 29, 39; Figs. 1-6) the diagnosis of carcinoma had later been changed to ameloblastoma, either at a new biopsy or at surgery. Cases 4, 19 and 45 (Figs. 8-12) had been classified and registered as malignant ameloblastomas.

Two of the 31 confirmed cases of ameloblastoma had been initially classified as other types of benign lesions, namely as squamous papilloma (31; Fig. 13) and salivary gland adenoma of the monomorphic or pleomorphic type (47; Fig. 14).

2. *Cases not re-confirmed as ameloblastoma.* Eighteen (18) of the 49 cases (36.7%) that had originally been registered as ameloblastoma were found upon re-examination to be benign lesions other than ameloblastoma (12 cases) or to be malignant tumors (six cases) (Table 1).

Of the 12 benign lesions, five were re-classified as benign odontogenic tumors, namely as adenomatoid odontogenic tumor (case 2), odontoma (case 12), calcifying odontogenic cyst (case 23), calcifying epithelial odontogenic tumor (case 35), and amelob-

TABLE 1 *Compiled Data of the 49 Ameloblastomas*

case no.	age (1)	sex (2)	location of tumor	dx at primary biopsy	dx at surgery	dx at present recurrences
1	43	F	mandible	A ¹⁾	A	follicular-cystic A
2	67	F	maxilla	A	A	adenomatoid odontogenic tumor
3	68	F	mandible	-4)	A	acanthomatous A
4	50	M	maxilla	malignant A	malignant A	pleuroform A
5	41	M	mandible		malignant A	poorly differentiated carcinoma
6	14	F	mandible	A	-4)	pleuroform A
7	39	M	maxilla	-	A	cyst with pseudocystlike contents
						hyperplasia
8	49	F	mandible	-	A	pleuroform A
9	30	M	mandible	fibrous ham- mameloma	fibrous ham- mameloma	ameloblastic adenoma-like tumor
10	26	M	maxilla		A	early A, cyst wall
11	15	M	maxilla		A	pleuroform A, cyst wall
12	9	M	mandible		fibrous ham- mameloma	adenoma
13	34	M	max. maxilla	A		total poly ps-sequestrum metaplasia
14	37	F	mandible	A	A	pleuroform A
15	36	F	mandible	A	A	pleuroform A
16	93	F	mandible	cyst	-	cyst
17	73	M	max. maxilla/ -maxilla	A	A	follicular A
18	53	F	oral mucosa base of skull	-	Amoebic A	crossed squamous
19	44	F	mandible	malignant A	malignant A	pleuroform follicular A
20	25	F	mandible		cyst A	karyocyst
21	43	M	mandible	-	A	pleuroform-acanthomatous A
22	61	M	mandible	A	A	follicular A
23	72	M	mandible		A	calcifying odontogenic cyst
24	60	M	maxilla	A		pleuroform A
25	50	M	maxilla	1 small carcinoma 2 A	A	pleuroform-follicular A
26	31	M	mandible	osteolytic	A	papilloma with cysts
27	18	F	mandible		A	follicular A, wall of karyocyst
28	83	F	mandible	A	A	cystic A
29	44	M	maxilla	carcinoma	A	pleuroform A
30	54	M	mandible	A	A	follicular A
31	28	M	maxilla	cytology papilloma	acanthomatous A	acanthomatous A
32	81	M	maxilla	A		pleuroform A
33	81	F	maxilla		A	cyst with pseudocystlike contents by periphery
34	61	M	maxilla	A	A	pleuroform A
35	38	F	mandible	A	A	calcifying epithelial odontogenic tumor
36	35	M	mandible	A	A	pleuroform A
37	19	F	mandible	-	cystic A	cystic A
38	17	F	base of skull	malignant tumor	malignant tumor	calcifying ameloblastic tumor
39	76	F	maxilla	carcinoma	A	acanthomatous A
40	74	M	mandible	A	carcinoma	carcinoma
41	74	M	mandible		A	acanthomatous A
42	65	F	maxilla	malignant A	malignant A	odontogenic carcinomatous cystic A
43	52	M	mandible	A	A	
44	51	F	mandible	A	-	pleuroform A, cyst wall
45	31	M	mandible		malignant A	pleuroform A, cyst wall
46	2	F	mandible	-	A	ameloblastic fibroma
47	89	M	mandible	1 adenoma 2 A	A	pleuroform A
48	23	F	mandible	A	A	follicular-pleuroform A
49	67	M	base, chin lower	-	Amoebic A	epithelial cell carcinoma

1) Age at registration in Cancer Registry

2) F = female, M = male

3) ameloblastoma

4) indicates no pre-operative biopsy and surgical specimen histologically not standard respectively

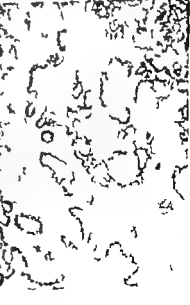
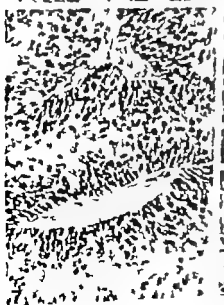
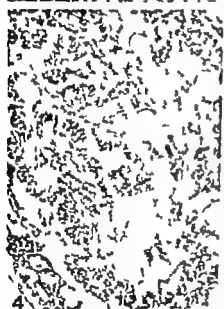


Fig 1 2 Case 25 Pleiform-follicular ameloblastoma. Biopsy diagnosed as basal cell carcinoma. It shows interdigitating cords of darkly stained, basallike cells with numerous epithelial buds, exhibiting a distinct palisading. These features are similar to those of basal cell carcinoma of skin. In deeper areas (Fig. 2) the lesion exhibits the features of a follicular ameloblastoma, with a distinct palisading.

Fig 3 4 Case 29 Pleiform ameloblastoma. Biopsy diagnosed as an epidermoid carcinoma. The connective tissue is permeated by interdigitating cords of epithelium, closely associated with the surface epithelium. The epithelial cords exhibit a characteristic palisading as well as numerous buds. At higher magnification (Fig. 4) the central part of the cords and buds are seen to be composed of closely packed polyhedral cells, with a characteristic cylindrical form of the peripheral cells.

Fig 5 6 Case 39 Acanthomatous ameloblastoma. Biopsy diagnosed as carcinoma. Irregular cords and strands of epithelium grow in the connective tissue closely associated with the surface epithelium. The peripheral cells are palisaded and mostly cylindrical. The central portions of some of the strands show extensive squamous metaplasia, with keratin pearls. At the growing edge of the lesion, (Fig. 6) isolated follicles exhibit squamous metaplasia.

Fig 7 Squamous cell carcinoma of gingiva. Part of biopsy showing irregular broad sheets of epithelium bordered by palisaded, cylindrical cells, superficially resembling an ameloblastoma. At closer examination, the palisaded as well as the centrally located cells lack the regularity normally associated with ameloblastoma. Less differentiated areas were also seen in this lesion, which was clearly originating from the gingival epithelium.

Fig 8 Case 4 Pleiform ameloblastoma. Biopsy diagnosed as a malignant ameloblastoma. Cell-rich strands and cords of epithelium grow in fibrovascular stroma. Numerous buds are observed at the growing edge, mostly with palisaded cylindrical cells. The central portions of the epithelium contain closely-packed cells with oval and slightly vesicular nuclei. No pleomorphism or mitotic figures can be seen.

Fig 9 10 Case 19 Pleiform-follicular ameloblastoma. Biopsy diagnosed as malignant ameloblastoma. Epithelial masses are permeating the connective tissue. Palisading of cubical-cylindrical cells is clearly seen. At the growing edge of the lesion, (Fig. 10) follicular structures appear with features typical of a follicular ameloblastoma, but there are also discrete and somewhat irregular sheets of epithelium, with squamous changes. No cell atypias are observed.

lastic fibroma (case 46). Four cases were designated as epithelial jaw cysts (7 16 20 33 Figs. 15 16 17). On the basis of the clinical data, case 18 was considered to be a cranio-pharyngioma rather than a jaw ameloblastoma (fig. 18). No ameloblastoma could be identified in the available material of cases 26 and 13 which were classified as a papilloma with epithelial dysplasia (Fig. 19) and nasal polyps with nonspecific inflammation and squamous metaplasia (Fig. 20).

Six of the 18 cases (5 9 38 40 42 49) that could not be classified as ameloblastoma upon re-examination, were found to exhibit histologic signs of malignancy. Three of these cases (5 40 49 Figs. 21-24) had originally been considered as benign or malignant ameloblastoma. Case 9 had originally been classified as a fibroadenomatous ameloblastoma. This case was included by Pindborg (1970) as an example of an ameloblastic odontosarcoma (see Fig. 434 in Pindborg's book). In case 38 classified as a malignant mesenchymal tumor at the base of the skull, ameloblastoma had been mentioned only as a remote possibility at biopsy and the available material contained no ameloblastomatous elements (Fig. 25). Upon re-examination, case 42 was found to be a most unusual tumor composed of pleomorphic epithelial elements growing within a cellular stroma, which also exhibited atypias (Figs. 26 27). We interpreted this as a mixture of carcinoma and sarcoma (odontogenic carcinosarcoma).

No adamantinoma of long bones had been reported to the Swedish Cancer Registry during the period 1958-1971.

Incidence, Age and Sex Distribution (Table 1 2 and 5)

The total number of tumors that fulfilled the histopathologic criteria of ameloblastoma upon re-examination of the 49 cases registered as ameloblastoma in Sweden during the period 1958-1971 was 31 (63.3%). The 31 tumors constituted 3.2% of all the 969 bone tumors and 43.7% of all the 71 skull and face tumors registered during the same period of time in Sweden.

The primary diagnosis of ameloblastoma had been made at the age of 40 years or later in 22 of the 31 reconfirmed cases (71%). The age distribution is shown in table 5. As to specific age incidence, there were 175 times more males than females in the older age group (14 and 8 respectively) whereas only a slight sex difference was evident in the younger age group (four males and five females). There was an overall predominance of males to females corresponding to 1.4:1 (18:13).

In the 49 registered cases of ameloblastoma, the

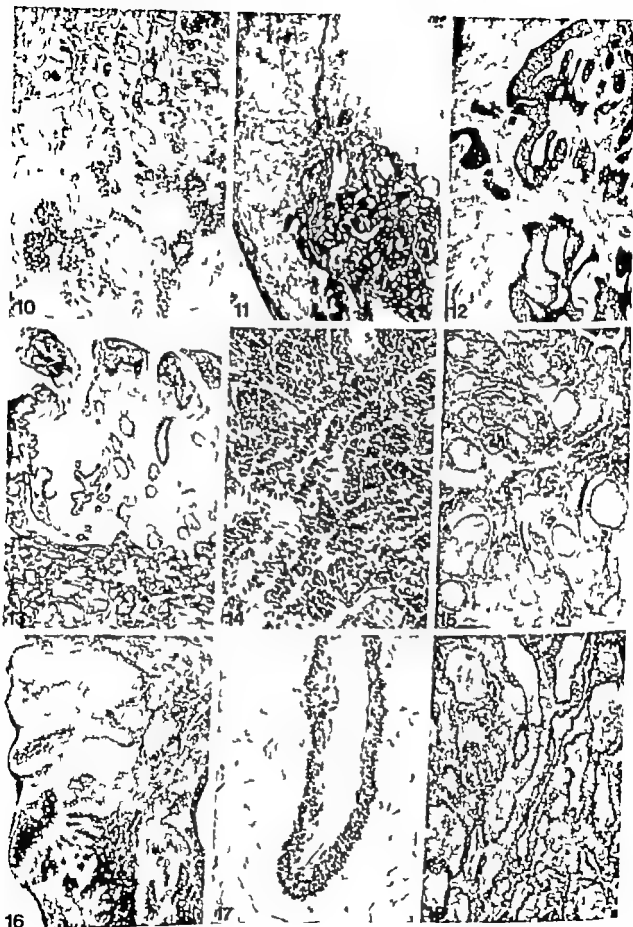


Fig 11 12 Case 45 Plexiform ameloblastoma. cyst wall. Part of locally excised cyst wall diagnosed as anagiant ameloblastoma, showing cyst epithelium and ameloblastoma developing in cyst-lining. Cords and nests of ameloblastoma epithelium are present in the cyst wall (Fig 12), apparently without connection with the cyst-lining epithelium.

Fig 13 Case 31 Acanthomatous ameloblastoma. Papillomatous lesion at the site of a previous extraction, diagnosed by cytology as a papilloma. The surface epithelium shows finger-like projections. Nests and cords of epithelium grow in the underlying connective tissue. A characteristic palisading of cylindrical cells was seen and the central areas of many small follicles showed a vascular pattern. In the anastipating strands the cells were closely packed and they showed squamous metaplasia with only a little tendency to keratinization.

Fig 14 Case 47 Plexiform ameloblastoma. Biopsy diagnosed as a salivary gland adenoma. Closely-packed cords and trabeculae of epithelium grow throughout the connective tissue. Palisading of cubical or cylindrical cells is apparent. In some areas, the cords are double layered. Nests of epithelium are budding off from the cords and the closely-packed cells exhibit small vacuolar nuclei. No whorling can be observed, see also Fig 28.

Fig 15 Case 7 Pseudoepitheliomatous hyperplasia of cyst-lining epithelium. Surgical specimen diagnosed as ameloblastoma. In nests and cords of epithelium in connective tissue of cyst wall, a slight tendency of palisading is seen as well as a tendency to keratinization. These epithelial changes are occasionally observed in jaw cysts, they should not be confused with an ameloblastoma.

Fig 16 Case 33 Pseudoepitheliomatous hyperplasia of cyst-lining epithelium. Surgical specimen diagnosed as ameloblastoma. Thick, rounded or slender strands of epithelium extend into inflamed cyst wall. Due to tangential sectioning, epithelial islands appear without apparent contact with cyst epithelium. The epithelium exhibited a slight atypia. These epithelial hyperplasias are occasionally seen in inflamed jaw cysts, but they have no relationship with ameloblastomas.

Fig 17 Case 20 Odontogenic keratocyst. Surgical specimen, diagnosed as an ameloblastoma. The cyst-lining epithelium exhibits an even thickness, a distinct basal cell layer and a parakeratin.

Fig 18 Case 18 Craniofacial dysplasia, ameloblastoma type. Tumor at the base of the skull, slightly bulging into the cranial cavity behind the chiasm of the optical nerve of a 52 year-old woman. Diagnosed at autopsy as an ameloblastoma. Laterigrating strands and cords of epithelium are bordered by palisaded cylindrical cells. The stroma is vascular. No calcifications were observed.

youngest patient was two and the oldest 93 years old, with an average of 50.5 years. In the 31 reconfirmed cases of ameloblastoma, the corresponding figures were 15, 87 and 50.2 years, respectively.

The yearly incidence rate of ameloblastoma varied between 0.13 and 0.63 cases per 1 million people, except for the years 1966 and 1969 during which no ameloblastomas had been registered at all in Sweden (Table 2).

Primary localization (Table 1)

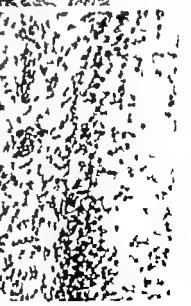
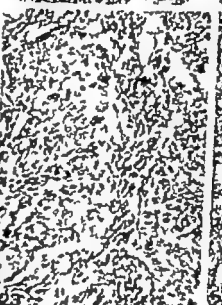
Twenty-three of the 31 proven cases of ameloblastoma (74%) were localized in the mandible, eight in the maxilla.

Primary diagnosis and diagnosis at re-examination (Table 3)

Clinically the 49 cases originally reported to the Swedish Cancer Registry as ameloblastomas during 1958-1971 had first appeared as accidental findings on X-rays, as asymptomatic swellings or occasionally as ulcerating or fungating growths. The initial removal of tissue for histopathologic examination had accordingly been performed by different routes. These are summarized in Table 3. Upon re-examination, 12 of 20 clinically cyst-like lesions fulfilled the histopathologic criteria of simple ameloblastoma (Table 3), whereas three were found to be bone cysts (16, 20, 33), one an adenomatoid odontogenic tumor (21), one an ameloblastic fibroma (46), one a carcinoma (5) and one an odontogenic keratocyst (23).

In 14 of the 49 registered ameloblastomas, the primary diagnosis had been made at biopsies of the oral or nasal mucosa (Table 3). In eight of these 14 cases, the diagnosis of ameloblastoma had been made at first examination, one 4 being described at the onset as a malignant ameloblastoma. In six of the 14 cases (25, 26, 29, 31, 39, 47), the final diagnosis of ameloblastoma had been settled only after an additional biopsy or at surgery.

Upon re-examination, ten of the 14 cases biopsied in the peripheral soft tissues were considered to represent simple ameloblastoma (Table 3), whereas the remaining four cases were classified as carcinomas (40, 49), papilloma (26) and squamous cell metaplasia (13). Of these ten reconfirmed ameloblastomas, six were of the plexiform, three of the follicular and one of the acanthomatous type. Only four of these ten cases (17, 24, 32, 34) had been initially classified as ameloblastoma, whereas four (4, 25, 29, 29) had been described as malignant tumors and the remaining two as benign tumors other than ameloblastoma (31, 47).



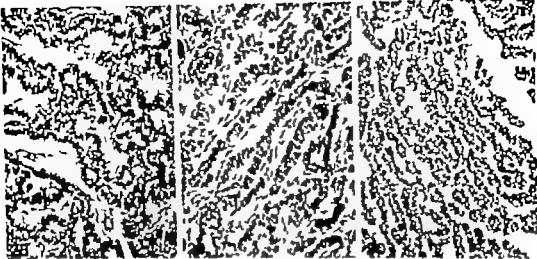


Fig 19 Case 26 Papilloma with dysplasia. Surgical specimen, diagnosed as ameloblastoma. From deeper portion of papillomatous tumor of the gingiva, with bone destruction. The basal type cell layer is prominent, owing to densely-packed cells with a homogeneously stained cytoplasm. The cells exhibit a slight pleomorphism, with an increased mitotic activity. Other areas showed more extensive dysplasia.

Fig 20 Case 13 Nasal polyps, squamous metaplasia. Small biopsy diagnosed as ameloblastoma. The inflamed connective tissue is covered by a respiratory epithelium exhibiting epidermoid and squamous metaplasia. The cuboid-cylindrical cells may give rise to suspicion of ameloblastoma cells or basal-squamous carcinoma.

Fig 21 Case 5 Poorly-differentiated carcinoma. Primary and recurrent tumor diagnosed as malignant ameloblastoma. Masses of densely-packed epithelial cells grow in a fibrous stroma. No clear-cut palisading is seen. The outline of the individual epithelial cells is difficult to discern. Vacuolization is prominent. No focal elements and no squamous metaplasia can be seen.

Fig 22 Case 40 Squamous cell carcinoma. Biopsy of oral mucosa, diagnosed as ameloblastoma. Well-demarcated, irregular sheets of epithelium appear in the connective tissue. There is no clear palisading of cells, no reticular pattern and no evidence of squamous metaplasia (keratin formation) in the epithelial masses.

Fig 24 Case 49 Squamous cell carcinoma of skin. Specimen excised at autopsy from lower region of chin and diagnosed as ameloblastoma. Elongated epithelial formations are seen in the lamina propria. There is some degree of palisading and the epithelium is clearly squamous, with areas of keratinization.

Fig 25 Case 38 Malignant mesenchymal tumor at the base of the skull. Ameloblastoma was considered as a remote possibility. Polymorphic cells grow diffusely in a fibrous stroma, and no ameloblastomatous structures can be seen.

Fig 26 Case 42 "Odontogenic carcino-sarcoma." Biopsy diagnosed as malignant ameloblastoma. A well-delimited epithelial island is seen in a cellular stroma, which differs from that of simple ameloblastoma. Very few or no fibre bundles can be discerned and the cells are haphazardly arranged. Owing to an extensive degree of vacuolization, the epithelium exhibits a reticular pattern. There is a slight tendency to palisading. **Fig 27** higher magnification of epithelial-mesenchymal border. The nuclei of the epithelial cells (right) are small and darkly stained in the centre, larger and more vesicular at the periphery where they also tend to aggregate in a palisaded pattern, reminiscent of that seen in a follicular ameloblastoma. The cellular mesenchymal tissue (left) exhibits irregularly-organized cells, which have elongated nuclei of a varying size and stainability. The cells tend to aggregate along the epithelial border in a fashion reminiscent of that seen in the developing tooth.

Fig 28 Trabecular adenoma of the oral mucosa. Soft tissue lesion in an elderly person, situated in the gingiva at the alveolar crest of the mandible, distal to the 2. molar. There was no bone involvement. The lesion is composed of cords and trabeculae of cylindrical epithelial cells arranged in double layers. Duct-like spaces are regularly formed. No myoepithelial cells can be seen. This lesion may be mistaken for an ameloblastoma, owing to the palisaded cylindrical cells, cf. **Fig 14** 29 and 30.

Fig 29 Case 14 Pleomorphic ameloblastoma. This is from a selected area of an ameloblastoma, characterized by interdigitating cords of epithelium, often composed of only two cell layers. The cells are cubical in appearance, with vesicular nuclei. There is little tendency to epithelial budding, and no ductlike elements are seen. Cf. trabecular adenoma. **Fig 28**

Fig 30 Case 34 Pseudofollicular ameloblastoma. In this variant, the epithelial cells are arranged in interdigitating cords which tend to form buds and small follicles. Many of the epithelial strands are double-layered. The cells are cubical, with small and slightly vesicular nuclei. Cf. trabecular adenoma. **Fig 28**

TABLE 2. Yearly Incidence of Ameloblastoma

year	registered cases No	A ¹⁾ reconfirmed upon reexamination, case No	number of reconfirmed cases of A ¹⁾ per 1 million people ²⁾
1958	1-67	1 3 4 6	0.54
59	7-12	8 10 11	0.40
60	13-15	14 15	0.27
61	16-20	17 19	0.27
62	21	21	0.13
63	22	22	0.13
64	23-27	24 25 27	0.39
65	28-31	28 29 30 31	0.52
66	-	-	-
67	32	32	0.13
68	33-38	34 36 37	0.38
69	-	-	-
70	39-46	39 41 43 44 45	0.63
71	47-49	47 48	0.25

1) A = ameloblastoma

2) cf Table 1

3) population figures according to the Swedish Cancer Registry

Malignancies and metastases (Table 4)

Malignancy had been considered initially in one of the 49 registered cases of ameloblastoma (18.4%), five of them being classified as malignant ameloblastomas (10.2%). The diagnoses of two cases (9.49), primarily classified as benign, were changed upon re-examination into malignant tumors and in case 40 the initial diagnosis of ameloblastoma had been changed into carcinoma before the final operation of the patient. Thus, malignancy was subject to discussion in 12 of the 49 registered cases of ameloblastoma (24%). These cases are summarized in Table 4.

Data were obtained from hospital files, including medical records of the patients at the time of examination or operative surgery. In several cases, information was also available about regular post operative check-ups. Metastases had been recorded in three of the 12 cases considered for malignancy (5.38.42). Upon re-examination, none of these three cases fulfilled the histopathologic criteria of ameloblastoma (see also »Histopathologic examina-

TABLE 3. Origin of Tissue and Diagnosis at Primary Examination and Re-examination

	case No. 7)	total	number of A ¹⁾ diagnosed at		number of malignant tumors diagnosed at	
			primary examin.	present re-examin.	primary examination 7)	present re-examination 7)
exploratory biopsy of bone lesion	9 14 21 30 35 36 42 43	8	8	5	1	2
excision of bone tumor	1 8 12, 45	4	4	3	1	0
excision or biopsy of cyst-like bone lesion	2 3 5 6 7 10 11 15 16 20 22 23 27 28 33 37 41 44 46 48	20	20	12	1	1
biopsy of oral or nasal mucosa	4 13 17 24 25 26 29 31 32, 34 39 40 47 49 3)	14	8	10	4	2
bone tumor found at autopsy	18	1	1	0	-	0
primary surgical specimen histologically not examined	19	1	-	1	1	0
unclear	38	1	1	0	-	1

1) A = ameloblastoma

2) for further details, see table 1 and »Results histopathological examination«

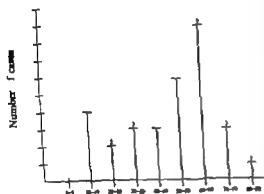
3) case 49 = skin specimen, autopsy

TABLE 4. *Malignancies and Metastases*

Case No	Initial dx	dx at re-examination	recorded metastases
4	malignant A ¹⁾	A	+
5	malignant A	carcinoma	
9	fibroepithelioid sarcoma	ameloblastic odontosarcoma	
19	malignant A	A	+
25	basal cell ca	A	
29	carcinoma	A	
38	malignant tumor	malignant mesenchymal tumor	
39	carcinoma	A	
40	A ²⁾	carcinoma	+
42	malignant A	odontogenic carcinosarcoma	
45	malignant A	A	
49	A	carcinoma	

1) A = ameloblastoma

2) changed to carcinoma before operation

TABLE 5. *Age distribution of the 31 reconfirmed cases of ameloblastoma at time of diagnosis*

those above), neither could any of the remaining cases of malignant ameloblastoma be histopathologically re-confirmed.

DISCUSSION

The 1% figure usually quoted in the literature as the occurrence of ameloblastomas of the jaws, is related to the total number of tumors and cysts of the jaws, including also all benign lesions. The latter lesions are not usually registered in the National Cancer Registry and the 1% frequency figure therefore reflects only the proportion of ameloblastomas relative to all jaw lesions submitted to selected pathology departments or other tumor service centres. Regarding the present results, they point at an incidence rate of ameloblastoma of about 0.3 cases per 1 million people per year in Sweden

The validity of these figures is difficult to evaluate. In order to determine the efficiency of reporting ameloblastomas to the Registry all ameloblastomas reported to the Cancer Registry by two of the largest hospitals in southern Sweden during the period 1958-1971 were recorded. These figures were correlated with the number of ameloblastomas actually diagnosed at these hospitals during the same period. The results showed that 5 out of a total of 10 ameloblastomas in one hospital and 5 out of 11 in the other hospital, i.e. about 50% of the ameloblastomas, had been reported to the Cancer Registry. Assuming that these figures are representative for the whole country correction for this assumption would bring the incidence rate of ameloblastoma in Sweden up to near by 0.6 cases per 1 million people, corresponding to an expected current total occurrence of about 5 cases per year.

It is known that the reporting of malignancy to the Swedish Cancer Registry is very efficient. The reason for the relative lack of recognition of compulsory registration of the ameloblastoma, is probably manifold. Even though the ameloblastoma has the reputation of being semi-malignant or locally invasive, the histologic features of the tumor are, however usually not such as to imply malignancy. This may at least partly explain the incomplete registration of ameloblastomas.

Many efforts have been made in the literature to analyze and discuss the ameloblastoma with respect to the site predilection, the sex distribution, the tissue of origin, and the relationship with odontogenic cysts and other odontogenic tumors. Our present results confirm earlier findings in this respect.

In the present study only about 63% of the 49

cases of ameloblastomas reported to the Swedish Cancer Registry during 1958-1971 were found to fulfil the histopathologic criteria of simple ameloblastoma. Among the cases not re-confirmed were a number of other benign odontogenic tumors, the histopathologic criteria of which have been defined in detail by WHO (Pindborg *et al* 1971). Ameloblastoma may appear as a predominantly cystic lesion, which may partly explain why several cases of epithelial jaw cysts had been primarily diagnosed as ameloblastoma. The precise diagnostic criteria concerning ameloblastoma and dental cysts have been delineated by *Vickers and Gorlin* (1970) who concluded that palisading with polarization, hyperchromatism of basal cell nuclei and vacuolization appearing together in the cyst epithelium suggest the interpretation of early ameloblastoma. So-called sprouting or budding and protruding epithelial islands, which may be seen in the case of epithelial hyperplasia in chronically inflamed dental cysts or epithelial extensions of odontogenic keratocyst linings, should not be considered neoplastic unless the epithelial protrusions also exhibit unequivocal features of ameloblastoma. Epithelial proliferations compatible with ameloblastoma may also be present in a wall of a cyst which is otherwise indistinguishable from routinely encountered dental cysts (*Vickers and Gorlin* 1970).

The question of malignancy in ameloblastoma was found to be a matter of controversy in several of the present cases and this matter has also been of much concern in the literature. *Carr and Halperin* (1968) reviewed all reports of malignant ameloblastoma during the period 1953-1966. They were able to collect 22 cases but found only 5 to be acceptable. *Gall *et al** (1975) were not able to find more than 10 previously-confirmed cases of malignant ameloblastoma, and among the cases published before 1966 only 2 of 5 cases listed by *Carr and Halperin* (1968) were accepted. All of the 10 cases listed by *Gall *et al** (1975) had also been included by *Herceg and Harding* (1972) as proven cases of malignant ameloblastoma but in addition to these 10 cases, *Herceg and Harding* had listed 9 more of Japanese origin. Eight of these Japanese cases had been published prior to 1966 but none of them was included by *Carr and Halperin* (1968) in their collection of malignant ameloblastomas.

From this short literature review it seems that there is no unanimous opinion about the criteria of malignant ameloblastoma. According to *Pindborg* (1970), an ameloblastoma should be called malignant only if it demonstrates metastases. The accounts of metastasizing ameloblastomas do however frequently contain areas of uncertainty (*Seward *et al** 1975). Much of this uncertainty

relates to the fact that many of the cases classified as malignant ameloblastomas have been subject to years of tissue manipulation and various types of treatment before showing sign of metastasis (*Gorlin *et al** 1961 *Smith* 1968). All these treatment procedures may also have favored aspiration of tumor fragments (*Hopson and Littlewood* 1972).

If metastases was the only criteria of malignant ameloblastoma, such a tumor could only be diagnosed in retrospect, unless metastasis was present at the time of discovery of the primary tumor. In the primary evaluation of the tumor histopathologic criteria of malignant ameloblastoma thus become highly significant. In this respect there is unfortunately some doubt in the literature about the possibilities of establishing the diagnosis of a malignant ameloblastoma histologically. According to *Gorlin *et al** (1961) ameloblastomas which will metastasize can simply not be histologically differentiated from non-metastasizing tumors. A more definitive view was taken by *Smith* (1968), who concluded that the ameloblastoma is a genuinely benign tumor which never metastasizes unless the tumor has been subjected to unusual over-stimulation.

We found in the present study that almost 25% of the reviewed cases were subject to discussion as regards malignancy either initially or upon re-examination, and that those ameloblastomas involving the oral soft tissues seemed to be particularly apt to cause diagnostic difficulties. This may partly be due to the fact that, in the peripheral location, the ameloblastoma tends to grow in a trabecular and cellular rather than in a follicular or cystic pattern, with signs of locally invasive growth. These features should not necessarily give rise to suspicion of malignancy. These tumors may however be indistinguishable from basal cell carcinoma of the skin (*Happle* 1973 *Simpson* 1974 *Lerchin and Rahbari* 1975) and may not be definitely differentiated from basal cell adenoma until the examination of the surgical specimen (cf Fig 28 and Figs 14, 19 and 39 *Kleinasser and Klein* 1967 *Evans and Cruickshank* 1970 *Batsakis* 1972, 1974 *Thackray and Lukas* 1974). Furthermore, in the peripheral location, simple ameloblastoma exhibiting squamous metaplasia may be mistaken for a squamous carcinoma exhibiting palisading of cells. The combination of squamous metaplasia and distinct palisading and a uniform size and texture of the cells and nuclei in the centre of the epithelial islands should rule out the carcinoma (cf Fig 7 and Figs 3, 4, 5, 6, 22 and 23).

Finally we could not re-confirm any of the diagnoses of malignant ameloblastoma. This may partly be explained by a previous lack of well-defined

criteria of malignant ameloblastoma. By current definition (Gorlin 1970 Pindborg et al 1971), such a tumour either shows metastases and /or exhibits cytologic features of malignancy together with unequivocal histologic features of ameloblastoma.

The authors are indebted to Dr Flim Pretorius and Dr Nils H. Sørensen for constructive criticism of the manuscript.

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Finally we could not re-confirm any of the diagnoses of malignant ameloblastoma. This may partly be explained by a previous lack of well-defined

«SMALL INTESTINAL TYPE» AND «COLONIC TYPE» INTESTINAL METAPLASIA OF THE HUMAN STOMACH

and Their Relationship to the Histogenetic Types of Gastric Adenocarcinoma

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Stubbe Teglbjerg, P. & Overgård Nielsen, H. «Small intestinal type» and «colonic type» intestinal metaplasia of the human stomach, and their relationship to the histogenetic types of gastric adenocarcinoma. *Acta path. microbiol. scand. Sect. A*, 86 351-355 1978.

Intestinal metaplasia in the non-tumour bearing parts of the gastric mucosa was demonstrated in 4 cases in a material consisting of 27 consecutive patients operated for carcinoma of the stomach. Two main histochemical types of intestinal metaplasia could be identified: 1) «small intestinal type» which was present in all 24 cases, and which was by far the most dominating type. 2) «Colonic type» which occurred in small foci in the gastric mucosa of 11 cases, and which was significantly more frequent in stomachs bearing tumours of presumed intestinal histogenesis, than in those bearing tumours of presumed non-intestinal histogenesis.

Key words: Stomach, human, intestinal metaplasia, colonic metaplasia.

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Received 23.1.78 Accepted 3.11.78

Within later years interest in intestinal metaplasia of the gastric mucosa, as the origin of carcinoma of the stomach, has increased considerably (5, 6, 7, 8, 9, 11, 12, 13, 14, 15, 16). Main histochemical studies (2, 3, 4, 17) have shown qualitative differences between mucin of the small intestinal mucosal goblet cells and the mucin of the goblet cells of the colonic mucosa. The object of the present work has been, firstly to attempt to identify histochemical types of intestinal metaplasia of the stomach having differences analogous to those existing between normal colonic and small intestinal mucosa. Secondly to determine whether possible different types of intestinal metaplasia of the gastric mucosa occur with the same frequency in stomachs which are the site of tumours of presumed intestinal histogenesis and stomachs bearing tumours presumed to be of non-intestinal histogenesis.

MATERIAL AND METHODS

Twenty-seven consecutive patients operated for carcinoma of the stomach comprised the material. The operative specimen was collected from the operating theatre, cut along the greater curvature, pinned and fixed for 48 hours in 4% formalin. Longitudinal sections were taken from the mucosa of the greater and lesser curvatures as well as from the anterior and posterior walls along the whole length of the stomach. Further four sections were taken from the tumour for histological examination. The material was embedded in paraffin, cut and stained using haematoxylin-eosin, alcian blue pH 2.6-PAS (10) and periodicate-borohydride/KOH/PAS. The periodicate-borohydride/KOH/PAS method demonstrates O-acetylated sialic acids in epithelial mucosa (2). This type of mucin is only produced in epithelial cells of the lower gastrointestinal tract of man under normal circumstances, i.e. the caecum, colon or rectum (or the terminal ileum immediately adjacent to the caecum) (3). Sections from normal small intestinal and colonic



mucosa were also stained in the same manner and used for control

The tumours were classified blindly by one of the authors (PST) who had no knowledge of the sections from the non-tumour bearing parts of the gastric mucosa. The tumours were classified as «intestinal cell carcinoma» (IC) «pyloro-cardiac gland carcinoma» (PC) «mucus cell carcinoma» (MC) or as unclassifiable, according to previously published criteria (18)

The occurrence of the below mentioned two types of intestinal metaplasia in the non-tumour bearing parts of the gastric mucosa was evaluated blindly by the other author (HON). He had no knowledge of the sections from the tumour

Fisher's exact test was employed in the statistical evaluation

RESULTS

Seven tumours were classified as IC, six as PC, thirteen as MC and one was unclassifiable. In the majority of the stomachs, a total of twenty four intestinal metaplasia was found in the mucosa (Table 1). The majority of the goblet cells in the intestinal metaplasia were stained purple by the alcian blue pH 2.6 PAS staining (Fig. 1) but were not stained by the periodate-borohydride/KOH/PAS method (Fig. 2). Intestinal metaplasia of this type, termed in the following «small intestinal type» was present in all the twenty four stomachs with intestinal metaplasia, and was by far the most dominating type. All the goblet cells in the control sections from normal small intestinal mucosa showed the same reaction to staining (Fig. 5 and 6).

In eleven cases, scattered foci of intestinal metaplasia were also seen, which reacted differently

to the staining. The goblet cells in these areas were light blue after staining by the alcian blue pH 2.6-PAS method (Fig. 3). Further the goblet cells in these areas were stained intensely red by the periodate-borohydride/KOH/PAS method (Fig. 4). This type of metaplasia is termed in the following «colonic type». Control sections from normal colonic mucosa showed the same reaction to staining (Fig. 7 and 8).

Further scattered intestinal metaplasia with goblet cells stained weak red following periodate-borohydride/KOH/PAS staining were seen, however these were purple after staining with alcian blue pH 2.6-PAS. Such areas were not considered as being of the «colonic type» of intestinal metaplasia.

All the stomachs, which were the site of tumours classified as IC, contained foci of «colonic type» of metaplasia, whereas this type was only present in one case of PC and in three cases of MC (Table 2). Intestinal metaplasia of the «colonic type» occurred significantly more frequently in cases of IC than PC ($p < 0.01$) and MC ($p < 0.01$) (Table 2).

TABLE 1 The Occurrence of Intestinal Metaplasia of Both the «Small Intestinal Type» and «Colonic Type» in the Non-Tumour Bearing Parts of the Gastric Mucosa

	IM	
	-	+
IC	0	7
PC	1	5
MC	2	11
Unclass	0	1
Total	3	24

Unclass = Unclassified, IM = Intestinal metaplasia.

Fig. 1 «Small intestinal type» of intestinal metaplasia of the stomach. The mucin of the goblet cells is stained purple. Normal gastric mucosa has mucin granules which stain red. Compare to fig. 3 (Alcian blue pH 2.6-PAS, $\times 100$).

Fig. 2 The same area as in fig. 1. All the goblet cells are unstained. Compare to fig. 4 (Periodate-borohydride/KOH/PAS, $\times 100$).

Fig. 3 «Colonic type» of intestinal metaplasia of the stomach. The mucin in the majority of goblet cells is stained light blue. A few areas of «small intestinal type» of metaplasia can be seen (arrow) (Alcian blue pH 2.6-PAS, $\times 100$).

Fig. 4 The same area as in fig. 3. The goblet cells stained light blue in fig. 3 are intensely red. (Periodate-borohydride/KOH/PAS, $\times 100$).

Fig. 5 Normal small intestine. The goblet cells are stained purple. Compare to fig. 1 (Alcian blue pH 2.6-PAS, $\times 100$).

Fig. 6 The same area as in fig. 5. The goblet cells are unstained. Compare to fig. 2 (Periodate-borohydride/KOH/PAS, $\times 100$).

Fig. 7 Normal colon. The goblet cells are stained light blue. Compare to fig. 3 (Alcian blue pH 2.6-PAS, $\times 100$).

Fig. 8 The same area as in fig. 7. The goblet cells are intensely red. Compare to fig. 4 (Periodate-borohydride/KOH/PAS, $\times 100$).

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TABLE 2 The Occurrence of «Colonic Type» of Intestinal Metaplasia in the Non Tumour Bearing Parts of the Gastric mucosa

	«Colonic Type» IM		
	-	+	Total
IC	0	7	7
PC	5	1	6
MC	10	3	13
Unclass	7	0	7
Total	16	11	27

IC compared to PC $p < 0.01$ IC compared to MC $p < 0.01$

Three tumours of the type MC as well as the unclassifiable tumour contained mucin which was stained intensely red by the periodate-borohydride/KOH/PAS method. Intestinal metaplasia of the «colonic type» did not occur in these stomachs. The remainder of the tumours of the types MC, as well as all of the IC and PC tumours were unstained by this method.

DISCUSSION

The present investigation demonstrates that two types of intestinal metaplasia occur in the stomach. The one type «small intestinal type» has goblet cells which are stained in a similar manner to those of the small intestine by periodate borohydride/KOH/PAS (3) and alcian blue pH 2.6-PAS methods (4, 17). Intestinal metaplasia of the «small intestinal type» was, in all the cases examined, present when intestinal metaplasia occurred in the gastric mucosa, and was the most common type.

The other type «colonic type» stains in a similar manner to normal colonic mucosa by these methods (2, 3, 4, 17). This type occurs in scattered foci and appears to be significantly associated with IC.

It is thus a tempting hypothesis, that intestinal metaplasia of the «colonic type» should have a certain premalignant potential whereas intestinal metaplasia of the «small intestinal type» should merely be of a reactive character without such a premalignant potential. A solution to this problem would require a prospective material followed by multiple biopsies.

In this respect it would be interesting to

determine the type of intestinal metaplasia in stomachs removed owing to a benign ulcer of the body of the stomach. It has unfortunately been impossible for us to obtain a large material of this type, as resection is rarely carried out for this disease in this country. However we have seen three cases from which we have taken histological sections, using the above technique, and in all three cases found intestinal metaplasia of the «small intestinal type» but no metaplasia of the «colonic type».

Abe *et al* (1) demonstrated that in cases of chronic gastritis, two types of intestinal metaplasia could be found in the stomach, «small intestinal type» and «colonic type», as evaluated from the staining for the enzymes, leucin-aminopeptidase and alkaline phosphatase, both of which are present in the mucosa of the small intestine, but not in stainable amounts in the normal mucosa of the colon. They found that «small intestinal type» of metaplasia was the most frequent and widespread type, which is analogous to our findings. Abe *et al* did not correlate these two types of metaplasia to types of carcinoma of the stomach.

Fifteen per cent (4/27) of the tumours in our material all without «colonic type» intestinal metaplasia of the mucosa, contained mucin which stained following periodate-borohydride/KOH/PAS method. Culling *et al* (3) found that one out of ten gastric carcinomas were stained by this method. Whether or not this can be of histogenetic significance is not clear.

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PRIMARY AMYLOIDOSIS OF THE URETER SIMULATING MALIGNANCY

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Willén, H. Primary amyloidosis of the ureter simulating malignancy. *Acta path. microbiol. scand. Sect. A*, 86: 357-359, 1978.

A 61-year-old woman with 7 years' history of pain, infections and haematuria developed right ureter stenosis which was suspected of being a tumour. Macroscopic examination of the ureter with stenosis showed primary amyloidosis. Although primary amyloidosis of the ureter is rare, it should be included in the differential diagnosis of ureter malignancy.

Key words: Amyloidosis, ureter neoplasia.

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Received 17.12.77 Accepted 10.10.78

Primary localized tumour-like amyloidosis in the ureter is very rare. Only few such cases have been reported (Johansson *et al.* 1964, Klotz 1975, Lee *et al.* 1976, Isobe *et al.* 1974, Magri *et al.* 1970, Takaha *et al.* 1971, Thomas *et al.* 1977).

CASE REPORT

A 61-year-old woman with a history of urinary infections in childhood, cystitis in 1970 and several attacks of haematuria and bacteriuria during 1971. In 1973 the patient got pain in the right flank. A tentative diagnosis of kidney colic was made. However, urography showed no stones. Cystoscopy revealed inflammation and oedematous changes of the right ureteral orifices (possibly after passed stone). During 1974 the patient was hospitalized three times with haematuria and attacks of pain in the right flank. In September 1974 catheterization of the right ureter revealed stenosis of the distal segment 1 cm above the uretero-vesical junction. During 1975 the patient was readmitted several times with haematuria, signs of inflammation and progressive right ureter stenosis. After the last attack of pain and haematuria in February 1977 an operation was suggested. A preoperative intravenous pyelogram demonstrated heavy distention of the right pelvis and the proximal part of the right ureter. The distal part of the ureter revealed advanced stenosis (Fig. 1). Because of the clinical history and the X-ray picture, cancer of the right ureter was



Fig. 1. Pyelogram demonstrating the stenosis of the distal ureter.

1976 Magri et al. 1970). The only case treated by conservative therapy showed a benign course (Johnson *et al* 1964)

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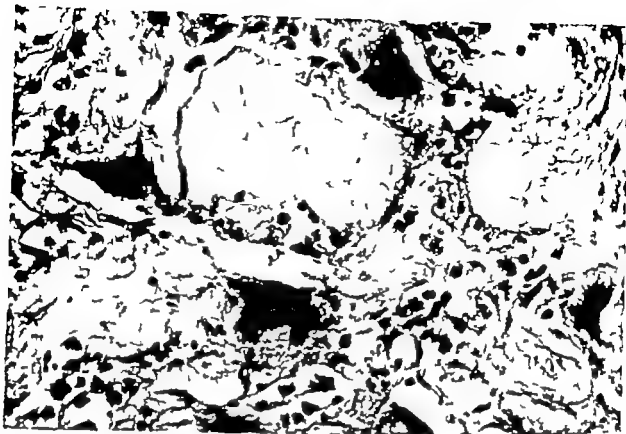


Fig 2 Giant cell reaction around amyloid deposits. Van Gieson. Orig. mag. $\times 160$

suspected. Other routine preoperative examinations were normal. A right nephro-ureterectomy including a rim of bladder tissue was performed. A pebbled stone was found in the bladder. The post-operative course was uneventful and all laboratory results were normal.

Macroscopic Appearance

The operation specimen showed a 10 cm long stenotic part of the distal ureter. The ureter wall was 1 cm thick. The ureter was adherent to the surrounding tissue, but there was no sign of infiltration.

Microscopic Appearance

The thickened part of the ureter revealed huge deposits of amyloid underneath a well-preserved mucosa. Lymphocytes and plasma cell infiltration, submucosal calcification and multinucleated giant cells (Fig. 2) around deposits of amyloid were observed. Alkaline Congo staining and examination by polarization microscope proved the diagnosis amyloidosis. The right kidney revealed only slight focal pyelonephritis. Alkaline Congo staining of slides from the kidney and non-stenotic parts of the ureter was negative for amyloid.

DISCUSSION

Primary localized amyloidosis is characterized by a tumour-like macroscopic appearance with no relation to other systemic disease and with local lymphocytes, plasma cells and giant cell reaction (Anderson 1971; Isobe *et al.* 1974; Klotz 1975). The

cause of primary amyloidosis is at present unknown, although many theories have been presented (Isobe *et al.* 1974). A local immunologic disturbance is supposed to be an important factor in the aetiology. In this case the development of the amyloidosis was most probably associated with a longstanding inflammatory state.

A few cases of primary amyloidosis of the ureter are on record (Isobe *et al.* 1974; Johnson *et al.* 1964; Klotz 1975; Lee *et al.* 1976; Magri *et al.* 1970; Takaha *et al.* 1971; Thomas *et al.* 1977). Most of the patients were over 50 years of age. The distal part of the ureter was often involved. Attacks of loin pain and haematuria are common symptoms. The X-ray picture of ureteral amyloidosis is often non-specific and cannot be distinguished from a true neoplasm. Submucosal calcifications in the renal pelvis and ureter detectable by urography were considered by some authors to be pathognomonic (Gardner *et al.* 1971; Takaha *et al.* 1971; Thomas *et al.* 1977). In this case calcium was observed only microscopically.

This case, together with the others, shows the necessity for including primary amyloidosis of the ureter in the differential diagnoses of ureter neoplasms. Tuberculosis and endometriosis in the female seem to be other alternative diagnoses.

Uretero-nephrectomy was performed in almost all cases (Gardner *et al.* 1971; Klotz 1975; Lee *et al.*

THE INCIDENCE AND CLINICAL RELEVANCE OF CHRONIC INFLAMMATION IN THE PANCREAS IN AUTOPSY MATERIAL

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Olsen, T. S. The incidence and clinical relevance of chronic inflammation in the pancreas in autopsy material. *Acta path. microbiol. scand Sect. A*, 86: 361-365 1978.

In 394 consecutive autopsies, tissues from the body of the pancreas showed chronic inflammation in 52 cases (13%); 32 were mild, 11 moderate and 9 severe. Only two of these cases had the clinical diagnosis chronic pancreatitis. The incidence of impervious plugs of protein in the ducts, dilated ducts and acinar ectasia was significantly higher when chronic inflammation was present. There was a significant higher incidence of chronic inflammation in the pancreas in patients with diabetes mellitus. No significant correlation was noted between chronic inflammation in the pancreas and cholelithiasis, previous cholecystectomy, peritonitis, gastric and duodenal ulcer, abdominal operations, ascites and liver metastases.

Key words: Pancreas, chronic inflammation, incidence, autopsy.

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Received 17 x 77 Accepted 15 in 78

Reports of symptomatic chronic pancreatitis are numerous (3, 6, 9, 10). Reports of the incidence and clinical relevance of chronic inflammation in the pancreas in autopsy material are however few. In addition the reported incidence is highly varying, the extreme figures being 0.18% (Edmondson *et al* 1949) and 66% (Stein & Powers 1958).

It therefore seemed worthwhile to investigate the incidence and clinical relevance of chronic inflammation in the pancreas in consecutive autopsies, using a combination of fibrosis and chronic inflammatory cell-infiltration as the histological criteria for the diagnosis.

MATERIAL AND METHODS

Routine sections from the body of the pancreas (one per case) were examined from 369 consecutive autopsies performed on adults more than 70 years of age during 1973.

The tissues were fixed in buffered formalin and stained with hematoxylin and eosin, and examined microscopically.

51 sections with primary or secondary neoplasms were excluded from the study because it was not possible to assess whether the inflammatory reaction around the neoplasm was of primary or secondary origin. A further 424 sections with marked autolysis were excluded because histological details were impossible to evaluate. The study presented is therefore based on 394 histological sections.

The intensity of fibrosis and chronic inflammatory cell-infiltration were each arbitrarily graded into 4 groups: 0 + + + +. The diagnosis of chronic inflammation was made when a combination of fibrosis and chronic inflammatory cell-infiltration was seen. These cases with chronic inflammation were divided into the three groups: mild, moderate and severe, according to the degree of fibrosis and chronic inflammatory cell-infiltration as seen in Table 1. More importance was attached to the degree of fibrosis than to the intensity of the cellular infiltrate when subjectively assessing the



Fig 3 Severe chronic inflammation of the pancreas (H&E $\times 50$)

dilatation of the pancreatic ducts and inspissated protein plugs in the ducts. These changes were correlated to the groups with and without chronic inflammation and tested for statistical significant difference using the chi square test.

The following findings were noted from the clinical history and autopsy: cholelithiasis, cholecystectomy, peptic ulceration of the stomach and duodenum, gastric operations, extra-abdominal operations, ascites, peritonitis, diabetes mellitus and liver metastases. These findings were correlated to the groups both with and without chronic inflammation and tested for statistical significant difference, using the chi square test and Fisher's exact test.

Information about alcohol consumption in the clinical records was inadequate and could not be used.

Routine tissue blocks were not taken from the liver and the incidence of fatty degeneration and cirrhosis of the liver was therefore unknown in the present material.

RESULTS

Chronic inflammation in the pancreas was seen in 52 cases (13%) 24 females and 28 males. It was not observed in any patient below the age of 40 years. The mean age for males with chronic inflammation was 67 years and for females 64 years.

32 cases were mild, 11 moderate and 9 severe as shown in Table 1. The degree of fibrosis and

chronic inflammatory cell-infiltration in these cases is also shown.

The clinical diagnosis chronic pancreatitis was made in 4 cases. In two cases chronic inflammation was present (one moderate and one severe). In the remaining two cases no signs of chronic inflammation were seen.

The incidence of inspissated protein plugs in the ducts, dilated ducts and acinar ectasia is seen in

TABLE 1 The Degree of Fibrosis and Chronic Inflammatory Cell-infiltration, in 52 Cases with Chronic Inflammation of the Pancreas

Degree of fibrosis	Degree of inflammatory cell infiltration		
	+	++	+++
+	30 mild	2 mild	0 moderate
++	11 moderate	5 severe	1 severe
+++	1 severe	1 severe	1 severe

The cases are subdivided into 3 groups: 32 cases with mild, 11 with moderate and 9 with severe chronic inflammation, according to the degree of fibrosis and chronic inflammatory cell-infiltration.



Fig 1 Mild chronic inflammation of the pancreas (HE x 50)

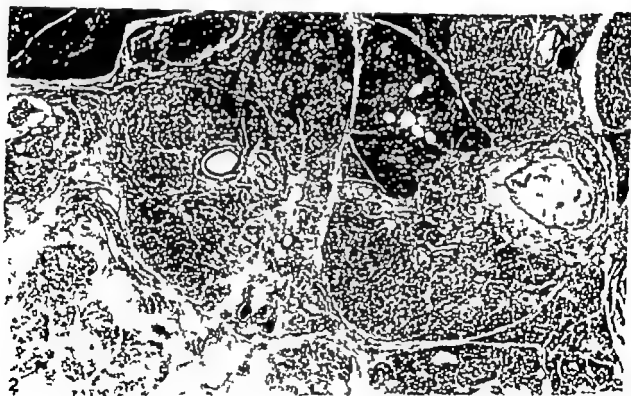


Fig 2 Moderate chronic inflammation of the pancreas (HE x 50)

degree of chronic inflammation as mentioned above. Examples of each of the 3 degrees of chronic inflammation in the pancreas are seen in Fig. 1, 2 and 3.

Small foci of fibrosis and scattered inflammatory cells

are very common in the pancreas and were not regarded as abnormal.

The presence or absence of the following pathological changes in the pancreas were recorded: acinar ectasia,

and severe in 9. Only two of these patients had a clinical diagnosis of chronic pancreatitis. In the remainder no pancreatic symptoms were reported in the clinical records. This study does not explain the discrepancy between the marked histological changes in the pancreas and the lack of reported symptoms in the clinical records. The 32 cases with a mild degree of chronic inflammation perhaps represent an entirely nonspecific reaction to a variety of conditions. Chronic pancreatitis can be a difficult diagnosis to establish clinically because of its varied symptomatology and the relatively complicated procedures used in its investigation. The evident inconsistency between the marked histological changes and the clinical symptoms in the 20 cases with moderate and severe chronic inflammation therefore indicates that chronic pancreatitis is perhaps underdiagnosed and/or often misdiagnosed.

Impacted protein plugs in the ducts, dilated ducts and acinar atrophy, were significantly more frequent in the cases with chronic inflammation than in those without. This supports the theory (Series 1972) that the first step in the development of chronic pancreatitis, at least in alcoholics, is impaction of protein material in the ducts resulting in obstruction, stagnation of secretions and consequent duct dilatation. However there were cases where chronic inflammation was present without these pathological changes, indicating that there are other pathogenetic possibilities for chronic inflammation in the pancreas.

The significantly higher incidence of chronic inflammation in the pancreas in patients with diabetes mellitus is noticeable but difficult to explain. In all cases, the chronic inflammation was mild. However the exocrine pancreatic function is slightly decreased in diabetes (Baron & Nabarro 1973).

Czernobitsky & Mikal (1964) in their study of interstitial pancreatitis found diabetes mellitus in 18.6% of the patients with interstitial pancreatitis and in 9.3% in those without. The difference was not significant because the material studied was too small.

Acutes has been reported as a rare complication of chronic pancreatitis (8) Czernobitsky & Mikal

(1964) found a significant correlation between acutes and interstitial pancreatitis in consecutive autopsy material. Such correlation was not apparent in this study.

There was no significant correlation between cholelithiasis, cholecystectomy, peritonitis, intra-abdominal operations, gastric operations and chronic inflammation in the pancreas. This supports the findings in other studies (4, 7).

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TABLE 2 The Number and Percentage of Sections with Impacted Protein Plugs Dilated Ducts and Acinar Ectasia in 52 Cases with and 342 Cases without Chronic Inflammation of the Pancreas

	+ Chr inflm 52 cases		+ Chr inflm 342 cases		Statistical significant difference
	Number	%	Number	%	
Impacted plugs in the ducts	13	25	37	11	$p < 0.01$ $\chi^2 = 6.95$
Dilated ducts	23	44	55	16	$p < 0.001$ $\chi^2 = 0.77$
Acinar ectasia	18	35	55	16	$p < 0.01$ $\chi^2 = 9.07$

Table 2 There was a significant higher incidence of these 3 pathological changes in the cases with chronic inflammation compared with those without.

The other nine clinical and autopsy findings are recorded in Table 3. There was a significant higher incidence of diabetes mellitus in the patients with chronic inflammation. All of these were in the group with mild chronic inflammation.

In the remainder there was no significant difference between the incidence of these findings in the groups with and without chronic inflammation in the pancreas.

DISCUSSION

This study is based on hospital autopsy material and does not represent a general population. 55% of the sections examined were excluded because of marked autolysis or neoplasms in the pancreas. The possibility that this may change the composition of the material cannot be excluded. The histological diagnosis is based on one section from the body of the pancreas. Thus the findings are not necessarily representative for the pancreas as a whole. Though this limits the scope of this study it gives some reliable impression of the incidence of chronic inflammation in the pancreas and its clinical relevance.

Because of the different criteria and method used in other studies on this subject it is not possible to compare the results directly. In the studies of Edmondson *et al.* (1949) and Becker (1963) the method is not sufficiently described to make a reliable comparison.

In this study the diagnosis of chronic inflammation was made only when combination of fibrosis and chronic inflammatory cell infiltration was seen. In studies on chronic interstitial pancreatitis Barret (1929) considered fibrosis sufficient criterion for the diagnosis whereas Stein & Powers (1958) accepted chronic inflammatory cell-infiltration as adequate. Chernobitsky & Wikat (1964) in their study of interstitial pancreatitis include both acute and forms termed healed interstitial pancreatitis.

In this study chronic inflammation was seen in 52 cases (13%) mild in 32 cases, moderate in 11

TABLE 3 The Number and Percentage of Nine Clinical and Autopsy Findings in 52 Cases with and 342 Cases without Chronic Inflammation in the Pancreas

Clinical & autopsy findings	+ Chr inflm 52 cases		+ Chr inflm 342 cases		Statistical significant difference
Cholelithiasis	9	17%	54	16%	NS $p > 0.05$
Previous cholecystectomy	9	17%	28	8%	-
Peptic ulceration of the stomach & duodenum	3	6%	26	8%	-
Previous gastric operations	6	12%	21	6%	-
Previous intraabdominal operation	21	40%	107	31%	-
Ascites	5	10%	24	7%	-
Peritonitis	3	6%	13	4%	-
Diabetes mellitus	10	19%	23	7%	$S 0.02 > p > 0.01$
Liver metastases	6	12%	47	14%	NS $p > 0.05$

Chi square test

Fischer's exact test

NS Not significant

S Significant

LIPOMATOSIS OF THE PANCREAS IN AUTOPSY MATERIAL AND ITS RELATION TO AGE AND OVERWEIGHT

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Olsen, T. S. Lipomatosis of the pancreas in autopsy material and its relation to age and overweight. *Acta path. microbial. scand. Sect. A*, 86: 367-373 1978

Lipomatosis, regarded as deposition of fat cells in the pancreas, was investigated and graded into four groups in 394 consecutive autopsies. In all necropsies except one fat cells were present, gr. 1 51% gr. 2 26% gr. 3 15% and gr. 4 8%. Age as well as overweight was significantly correlated to the grade of lipomatosis. The number of necropsies with gr. 3-4 lipomatosis was significantly less in the group with long venereal illness, indicating that the presence of fat cells in the pancreas is to some extent reversible. No evidence of pancreatic disease was mentioned in the clinical records of the patients with gr. 3-4 lipomatosis.

Key words: Pancreas, lipomatosis, age, obesity, autopsy.

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Received 17 x 77 Accepted 15 in 78

Lipomatosis, regarded as deposition of fat cells in the pancreas, is the most common histological change in the pancreas (Wallace & Ashworth 1942, Waters 1966). Despite this, it has received little attention. Most authors suggest the change to be associated with age and obesity (Bowd 1938, Wallace & Ashworth 1942, Dreiling 1961, Waters 1966), but no statistical verification is available.

The purpose of this study is to investigate the degree of lipomatosis in the pancreas, in histological sections from consecutive autopsies and investigate any correlation to age and overweight.

MATERIAL AND METHODS

During 1973 routine tissue blocks from the body of the pancreas (one per case) were taken from 869 consecutive autopsies on adults more than 20 years.

The tissues were fixed in buffered formalin and stained with haematoxylin and eosin and examined microscopically.

424 sections with marked atypias were excluded because histological details were impossible to evaluate. 51 sections with neoplasms were also excluded because it was not possible to decide whether the pathological changes in the surroundings of the neoplasms were primary or secondary.

The study presented, is therefore based on 394 histological sections.

The degree of lipomatosis was graded into 4. Grade 1 represents sections with few scattered fat cells in the exocrine parenchyma. Grade 4 represents sections with partial or total replacement of exocrine lobules by fatty tissue. Grade 2 and 3 were sections with fat cell content between grade 1 and grade 4.

The material was subdivided into 10-year age groups: 20-29, 30-39, etc. and correlated to the degree of lipomatosis. The correlation was tested for statistical significance by means of the contingency coefficient test (5).

By using a height-weight table (3) the material was subdivided into the following groups: weight below normal, normal and above normal and correlated to the degree of lipomatosis. The correlation was tested for statistical significance by means of the contingency

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The material was subdivided into 10 year age groups: 20-29, 30-39, etc., and correlated to the degree of lipomatosis. The correlation was tested for statistical significance by means of the contingency coefficient test (5).

By using a height-weight table (3) the material was subdivided into the following groups: weight below normal, normal and above normal and correlated to the degree of lipomatosis. The correlation was tested for statistical significance by means of the contingency

coefficient test. (In 17 cases, the information regarding weight and height was inadequate and this part of the investigation therefore includes only 377 autopsies).

The degree of lipomatosis was correlated to the

duration of the terminal illness, grouped as long terminal illness (≥ 2 month) and short terminal illness (< 1 month). The statistical difference was tested by means of the chi square test

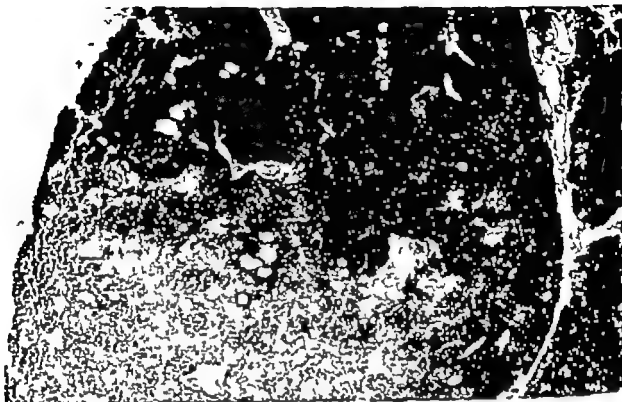


Fig 1 Grade 1 lipomatosis. (HE \times 50)



Fig 2 Grade 2 lipomatosis. (HE \times 50).

17 To demonstrate that long terminal illness results in low weight, the patients with weight below normal vs normal and above normal were divided into two groups according to the duration of the terminal illness. The

difference between these two groups was tested for statistical significance difference using the chi square test.

All cholangiograms from patients with grade 3-4 lipomatosis were examined for evidence of pancreatic disease.

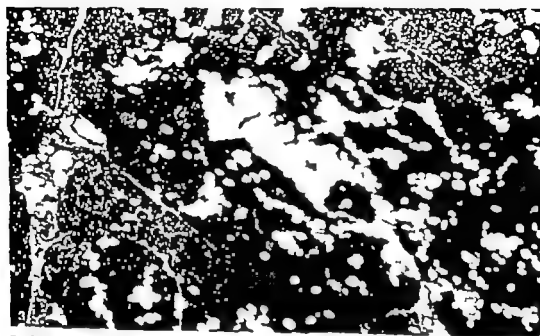


Fig 3 Grade 3 lipomatosis (H&E x 50).

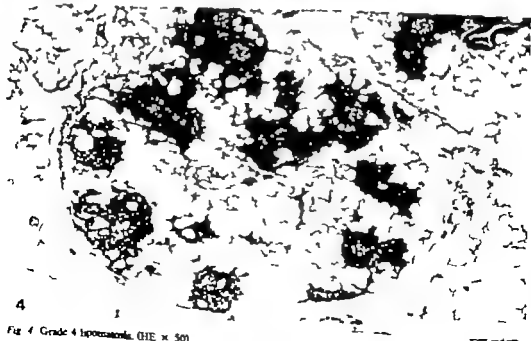


Fig 4 Grade 4 lipomatosis (H&E x 50)

RESULTS

The material presented includes necropsies from patients in the age range 20–96 years. 82% of the patients died in the 5th–8th decade of life.

In all necropsies except one (included in grade 1) fat cells were seen in varying numbers, from a few scattered cells, to areas of partial or total replacement of exocrine lobules, where only the islets of Langerhans were spared (Fig. 1–4).

Table 1 shows the number and percentage of necropsies with grade 1–4 lipomatosis, total and in the various age groups.

Fig. 5 shows the percentage of necropsies with gr 1–4 lipomatosis in each of the age groups. From this figure it appears that the degree of lipomatosis increases with increasing age. By means of the

contingency coefficient test (using the data in Table 1) this is verified statistically (Chi square = 64.08, $p < 0.001$ the contingency coefficient $C = 0.37$).

The number and percentage of necropsies with gr 1–4 lipomatosis in each of the weight groups below normal, normal and above normal is seen in Table 2. The distribution of necropsies with gr 1–4 lipomatosis in the 3 weight groups expressed as a percentage is seen in Fig. 6. The number of necropsies with gr 3–4 lipomatosis increases from the group, weight below normal to weight above normal and the number of necropsies with gr 1 lipomatosis decreases. Applying the contingency coefficient test on the data in Table 2 this is statistically verified. (Chi square = 13.24 $0.05 > p > 0.02$ the contingency coefficient $C = 0.18$).

Because the degree of lipomatosis increases with

Table 1 The Number and Percentage of Necropsies with Gr 1–4 Lipomatosis in Each of the Age Groups

Age group	Degree of lipomatosis								Total Number
	1		2		3		4		
	Number	%	Number	%	Number	%	Number	%	
20-29	5	100	0	0	0	0	0	0	5
30-39	15	88	1	6	1	6	0	0	17
40-49	32	80	6	15	1	3	1	3	40
50-59	36	64	17	30	2	4	1	2	56
60-69	42	47	20	22	14	16	13	15	89
70-79	42	39	30	28	24	22	11	10	107
80-89	27	36	27	36	14	19	6	8	74
90-99	0	0	3	50	3	50	0	0	6
Total	199	51	104	26	59	15	32	8	394

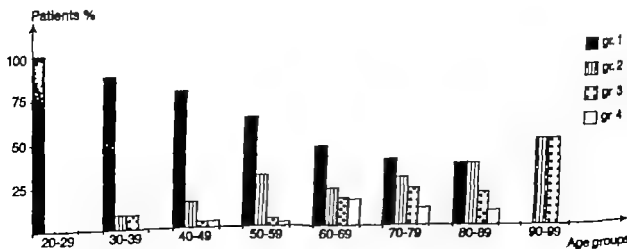


Fig. 5 The percentage of necropsies with gr 1–4 lipomatosis in each of the age groups.

Table 2 The Number and Percentage of Necropses with Gr 1-4 Lipomatosis in the Weight Groups below Normal, Normal and above Normal

Degree of lipomatosis	Weight						Total Number
	Below normal		Normal		Above normal		
	Number	%	Number	%	Number	%	
1	100	61	70	48	24	37	194
2	38	23	44	30	16	25	98
3	18	11	21	14	16	25	55
4	9	5	12	8	9	14	30
Total	165	100	147	100	65	100	377

increasing age, one may suggest that this result is due to a relatively greater number of patients with advanced age in the group weight above normal. Therefore the number of patients with weight below normal, normal and above normal in each of the age groups is tabulated in Table 3. Applying the contingency coefficient test on these data, no significant correlation between age and the 3 weight groups was found. (Chi square = 16,37 $p > 0.05$ the contingency coefficient $C = 0,20$).

The number of patients with long and short terminal illness correlated to the weight-groups below normal, normal and above normal is shown in Table 4. The number of patients with weight below normal is significantly more frequent in patients with a long terminal illness, indicating that

long terminal illness results in weightloss. (Chi square = 17.46 $p < 0,01$)

The number of patients with long and short

Table 3 The Number of Patients with Weight below Normal, Normal and above Normal in Each of the Age Groups

Age group	Weight			Total
	Below normal	Normal	Above normal	
20-29	4	1	0	5
30-39	8	6	2	16
40-49	21	13	6	40
50-59	26	16	11	53
60-69	29	37	19	85
70-79	39	51	13	103
80-89	34	23	13	70
90-99	4	0	1	5
Total	165	147	65	377

Table 4 The Number of Patients with Long and Short Terminal illness Correlated to the Weight Groups below Normal, Normal and above Normal

Duration of terminal illness	Weight			Total
	Below normal	Normal	Above normal	
Long	94	52	23	169
Short	71	95	42	208
Total	165	147	65	377

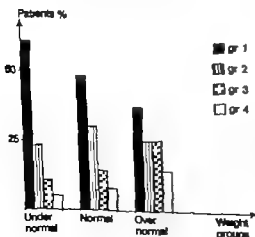


Fig 6 The percentage of necropses with gr 1-4 lipomatosis in the weight groups below normal, normal and above normal

Table 5 The Number of Patients with Long and Short Terminal Illness Correlated to the Degree of Lipomatosis

Duration of terminal illness	Degree of lipomatosis						Total
	1	2	1 + 2	3	4	3 + 4	
Long	101	54	155	20	9	29	184
Short	98	50	148	39	23	62	210
Total			303			91	394

terminal illness in relation to the 4 degrees of lipomatosis is tabulated in Table 5. There is a significant difference between the two groups, gr 1-2 lipomatosis and gr 3-4 lipomatosis, using the chi square test. The number of patients with long terminal illness is significantly less in the group gr 3-4 lipomatosis (chi square = 9.69, 0.01 > p > 0.001). This indicates that weightloss results in a reduction in the fat cell content of the pancreas.

No evidence of pancreatic disease was mentioned in the clinical records from patients with gr 3-4 lipomatosis.

DISCUSSION

This study is based on hospital autopsy material and does not represent a general population. 55% of the sections examined were excluded because of marked autolysis or neoplasms in the pancreas. The possibility that this may change the composition of the material cannot be excluded. The histological diagnosis is based on one section from the body of the pancreas. Thus the findings are not necessarily representative for the pancreas as a whole. Though this limits the scope of this study, it gives some reliable impression of the incidence of lipomatosis in the pancreas and its relation to age and overweight.

This study supports the results of other investigators (6, 7) that lipomatosis is the most common histological change in the pancreas. In all cases, except one, fat cells were present, and therefore this change must be considered a normal finding in hospital autopsy materials.

The significant correlation between increasing degrees of lipomatosis and increasing age, and the significant correlation between the degree of overweight and the degree of lipomatosis confirms other non-statistical verified reports (3, 6, 7).

This study gives no direct information about the cause of lipomatosis in the pancreas. Whether the degree of lipomatosis reflects a corresponding exocrine atrophy or simple fat infiltration without disappearance of exocrine tissue, is not quite clear. The fact that areas of fatty tissue with scattered

islets of Langerhans were seen in gr 3-4 lipomatosis, indicates however that the lipomatosis reflects previous loss of exocrine tissue. This assumption is supported by Walters (1966) and Baló (1929). Walters studied the exocrine rat-pancreas after ligation of the pancreatic duct. The end result of this experiment was degeneration and lysis of the acinocytes followed by fat cell replacement. Baló found the dry weight of the pancreas after extraction of fat to be 11-20 gm in the normal pancreas and 5-12 gm in the fatty glands indicating that atrophy of the pancreatic parenchyma had occurred.

Dreiling *et al* (1961) found subnormal exocrine pancreatic function in patients with obesity. The pancreatic function returned to normal after marked weight reduction. They suggested that the subnormal pancreatic function was due to fatty infiltration. They therefore concluded that the deposition of fat cells in the pancreas is reversible and that the existence of fat cells in the pancreas should be termed fatty infiltration rather than lipomatosis.

The results in this study indicate that the presence of fat cells in the pancreas is reversible. But this is only to a certain extent. First because the lipomatosis is increasing with increasing age, and second because gr 4 lipomatosis was present, in this study in emaciated patients with a long terminal illness. It therefore seems reasonable to maintain the term lipomatosis.

No evidence of pancreatic disease was mentioned in the clinical records from patients with gr 3-4 lipomatosis. As gr 4 lipomatosis reflects about 50% or more fatty tissue in the histological slides the pancreatic functional capacity must be great.

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Table 5 The Number of Patients with Long and Short Terminal Illness Correlated to the Degree of Lipomatosis

Duration of terminal illness	Degree of lipomatosis						Total
	1	2	1+2	3	4	3+4	
Long	101	54	155	20	9	29	184
Short	98	50	148	39	23	62	210
Total			303			91	394

terminal illness in relation to the 4 degrees of lipomatosis is tabulated in Table 5. There is a significant difference between the two groups, gr 1-2 lipomatosis and gr 3-4 lipomatosis, using the chi square test. The number of patients with long terminal illness is significantly less in the group gr 3-4 lipomatosis (chi square = 9.69, $0.01 > p > 0.001$). This indicates that weightloss results in a reduction in the fat cell content of the pancreas.

No evidence of pancreatic disease was mentioned in the clinical records from patients with gr 3-4 lipomatosis.

DISCUSSION

This study is based on hospital autopsy material and does not represent a general population. 55% of the sections examined were excluded because of marked autolysis or neoplasms in the pancreas. The possibility that this may change the composition of the material cannot be excluded. The histological diagnosis is based on one section from the body of the pancreas. Thus the findings are not necessarily representative for the pancreas as a whole. Though this limits the scope of this study it gives some reliable impression of the incidence of lipomatosis in the pancreas and its relation to age and overweight.

This study supports the results of other investigators (6, 7) that lipomatosis is the most common histological change in the pancreas. In all cases except one, fat cells were present, and therefore this change must be considered a normal finding in hospital autopsy materials.

The significant correlation between increasing degrees of lipomatosis and increasing age, and the significant correlation between the degree of overweight and the degree of lipomatosis confirms other non-statistical verified reports (3, 6, 7).

This study gives no direct information about the cause of lipomatosis in the pancreas. Whether the degree of lipomatosis reflects a corresponding exocrine atrophy or simple fat infiltration without disappearance of exocrine tissue, is not quite clear. The fact that areas of fatty tissue with scattered

islets of Langerhans were seen in gr 3-4 lipomatosis, indicates however that the lipomatosis reflects previous loss of exocrine tissue. This assumption is supported by Walters (1966) and Balo (1959). Walters studied the exocrine rat-pancreas after ligation of the pancreatic duct. The end result of the experiment was degeneration and lysis of the acinocytes followed by fat cell replacement. Balo found the dry weight of the pancreas after extraction of fat to be 11-20 gm. in the normal pancreas and 5-12 gm. in the fatty glands indicating that atrophy of the pancreatic parenchyma had occurred.

Drelling *et al* (1961) found subnormal exocrine pancreatic function in patients with obesity. The pancreatic function returned to normal after marked weight reduction. They suggested that the subnormal pancreatic function was due to fatty infiltration. They therefore concluded that the deposition of fat cells in the pancreas is reversible and that the existence of fat cells in the pancreas should be termed fatty infiltration rather than lipomatosis.

The results in this study indicate that the presence of fat cells in the pancreas is reversible. But this is only to a certain extent. First because the lipomatosis is increasing with increasing age, and second because gr 4 lipomatosis was present in this study in emaciated patients with a long terminal illness. It therefore seems reasonable to maintain the term lipomatosis.

No evidence of pancreatic disease was mentioned in the clinical records from patients with gr 3-4 lipomatosis. As gr 4 lipomatosis reflects about 50% or more fatty tissue in the histological slide the pancreatic functional capacity must be great.

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THE JUXTAGLOMERULAR APPARATUS IN A HUMAN KIDNEY WITH POLAR ARTERY STENOSIS

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Christensen, J. A., Meyer, D. S., Jakubowski, H. D., Neuenhöfer, J. & Bohle, A. The juxtaglomerular apparatus in a human kidney with polar artery stenosis. *Acta path. microbiol. scand. Sect. A*, 86: 375-381 1978.

Juxtaglomerular apparatuses (JGA) of a human kidney with stenosis of a polar artery (from a hypertensive 18-year-old male patient) were studied qualitatively and quantitatively on 2 μ thick serial sections from plastic embedded renal tissue. 11 JGA from juxtamedullary and 9 JGA from subcapsular cortical zones were photographed serially, the copies taped together and the cells and relationships within the JGA studied. On the copies the length of contact between the different juxtaglomerular structures and the basement membrane of the macula densa was measured. Furthermore we calculated the areas of surface contact and the macula densa basal area. In the juxtamedullary JGA affected by the polar artery stenosis all Goormaghtigh cells were transformed into epithelioid cells and the Goormaghtigh cell field was significantly larger than in the JGA of the subcapsular cortex, which obviously had been exposed to the systemic hypertension. The macula densa basal area was significantly greater in the juxtamedullary JGA than in the subcapsular JGA, but neither of these differed significantly from the macula densa in the normal JGA reported previously.

Key words: Juxtaglomerular apparatus, renal hypertension, juxtaglomerular contact areas, macula densa, basal area.

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Received 16 vii 1978 Accepted 16 viii 1978

Reduced renal perfusion pressure with or without reduced renal blood flow due to stenosis of the renal artery is regularly followed by stimulation of the juxtaglomerular cell complexes. This provokes an increased renin secretion with secondary hypoadosteronism and hypertension. Elevated renin activity in peripheral plasma is often found in cases of stenosis of the renal artery (5, 13, 18, 20, 21, 24, 25, 30, 35).

The JGA consists of the afferent arteriole, the efferent arteriole, macula densa (MD) and the Goormaghtigh cell field (Go cell field), which is

located between the hilar arterioles and the MD. The term juxtaglomerular cell complex (JGC-complex), (28) refers to the epithelioid cells in the arteriolar walls and the Goormaghtigh cells (Go. cells).

Today there is good evidence supporting the opinion that the renin by the kidneys is liberated from the granules in the epithelioid cells (10, 37).

It might therefore be anticipated that morphological studies on the JGA in renal hypertension would give results different from the JGA in the normal kidney.

This paper deals with a hypertensive patient with

mm Hg). Two years later he was admitted to the hospital for precise diagnosis of his hypertension, and a stenosed upper polar artery was found on the right side by renal angiography (Fig. 1). An attempt was made to correct the stenosis surgically but owing to technical difficulties during the operation, the kidney pole had to be resected. The postoperative period was uncomplicated and 2 weeks after the operation the renal angiogram was found to be normal with simultaneous filling of both kidneys.

9 months after the operation the patient was normotensive with normal kidney function. The main clinical data are summarized in Table 1.

MATERIAL AND METHODS

The removed kidney tissue was perfused in neutral 4% formaldehyde. The renal cortex was dissected into subcapsular and juxtamedullary stripes and cut into small blocks with edges 1.5–2 mm. These blocks were postfixed in OsO₄ and embedded in Epon 812.

2 μ m thick serial sections were cut on a Reichert Ultramicrotome Type Om U3 with glass knives. The series consisted of 100–200 consecutive sections which were Gomori-stained on slides.

All sections from 9 subcapsular and 11 juxtamedullary JGA were photographed with a Zeiss Photomicroscope at a final magnification of 370 \times . The JGA were orientated similarly in each section. The copies were taped together and serial inspection of consecutive copies then made it possible to distinguish the various juxtaglomerular structures.

On the copies we measured the extent of contact between the juxtaglomerular structures and the length of the MD basement membrane. Taking into consideration the magnification and the thickness of the sections, we calculated the areas of the surface contact between juxtaglomerular structures and the basal area of the MD.

The JGA were chosen according to the following criteria:

- 1) The whole JGA must be present in the series.
- 2) The arterioles had to be definitely identified.
- 3) The JGA should not be located marginally in the sections.

As normal reference data we used previously reported results from normal human kidneys. This material consisted of JGA from all parts of the cortex (table 2), (6).

The data were analysed statistically using the Wilcoxon-Test. We compared the subcapsular with the juxtamedullary JGA in the affected part of the kidney and these two groups with our data from normal human kidneys. Level of significance $p = 0.05$.

RESULTS

The JGA in the subcapsular and juxtamedullary cortical layers differed qualitatively very distinctly from each other. The subcapsular JGA looked quite normal although somewhat smaller than in the

normal kidneys (Fig. 2), whereas in the juxtamedullary JGA nearly all Go. cells were transformed into epithelioid cells (Fig. 3). Transformation of mesangial cells into epithelioid cells was not identified with the light microscope. The border between the metanephros and the Go. cells was set at the glomerular hilus where the parietal layer of Bowman's capsule continues into the visceral one (Fig. 3), (8). In the juxtamedullary JGA we found an increase of epithelioid cells in the hilar arterioles most pronounced in the afferent arterioles (Fig. 3).

The distal tubules showed variation in form as previously described for normal kidneys (6, 8).

The measurements revealed a strikingly enlarged Go. cell field in the juxtamedullary JGA, with significantly greater contact areas with MD and hilar arterioles than in the subcapsular and normal JGA (tab. 2 and 3).

The mean MD basal area was significantly larger in the juxtamedullary JGA (5822 μ m²) than in the



Fig. 2 JGA from the subcapsular cortex. Afferent arteriole on the right side, efferent arteriole to the left at the glomerular hilus. Between the arterioles the densely stained Goormaghtigh cell field. Above, the distal tubule with macula densa. The Goormaghtigh cells proceed continuously into the mesangial cells. Picric acid embedding, semithin section (2 μ), Gomori stain 570 \times .



Fig 1 Preoperative renal angiogram. Notice the stenosed upper polar artery with a poststenotic dilatation (Arrow)

stenosis of an upper polar artery and local hyperplasia of the JGA but with normal plasma renin activity (PRA). The opinion will be discussed that this constellation comprises too high a PRA (18-19) in relation to the patient's blood pressure.

CLINICAL FINDINGS

A white male patient was found to be hypertensive at the age of 16 years on routine examination. His systolic pressure was 170 mm Hg and the diastolic pressure 110 mm Hg (maximal values 180/120

TABLE I *Main Clinical Data*

	Preoperative	9 Months Postoperative
Blood pressure (mm Hg)	170/110	120/80
Total blood volume	7480	4716
Volume of erythrocytes (ml)	3140	1956
Plasma volume (ml)	4340	2760
PRA (ng Angiotensin I/ml/h)		
recumbent	0.9	1.1
walking	1.3	2.2
Creatinine-Clearance (ml/min)	100	125
Serum-Creatinine (mg %)	0.95	1.1
Fundus oculi	Grade 0-1 Hypertensive changes	No signs of hypertension

TABLE 3 Juxamedullary and Subcapsular JGA in the Stenosed Kidney Compared with Each Other and with Normal Reference Material (%)

		MD	Go/MD	Go/Art		MD/Art	
				As	As	As	As
Juxamedullary Subcapsular	JGA	++	++	++	++	++	++
Juxamedullary Normal	JGA	NS	++	++	++	Insufficient number with contact	
Normal Subcapsular	JGA	NS	++	++	++	++	++

++ significant ($p < 0.001$)NS not significant ($p > 0.05$)

For explanation of symbols, see Table 2

peripheral venous blood despite considerably enlarged JGA in the juxamedullary cortex as revealed by the morphometric study of the removed kidney pole.

Kortzmann *et al.* (23) and Lokumeier and Davis (24) were able to show under experimental and clinical conditions that increased PRA depends on negative sodium balance. This occurred only in the malignant phase of experimental hypertension, by sodium depletion or after administration of diuretics. It is, therefore, in the present case relevant to ask if there exists a discrepancy between the hypertension, the hyperplastic JGA in particular parts of the stenosed kidney pole and the normal PRA, or if these findings might be reconciled with our present knowledge of the renin-angiotensin-aldosterone system.

Tobies's hypothesis (38) that baroreceptors in the afferent arterioles are stimulated by a poststenotic decreased intravascular pressure and/or volume and that this stimulation in turn is followed by an increased renin liberation and angiotensin synthesis in the JGC-complexes, is supported by the morphometric and morphologic findings in the resected kidney pole. The juxamedullary JGA were clearly enlarged compared with previously reported findings in normal kidneys (6-7). This enlargement was caused particularly by the epithelioid transformation of the Go cells and the smooth muscle cells in the media of the arterioles.

This morphologic pattern is still more pronounced when comparing the juxamedullary JGA with the JGA in the subcapsular cortex, in which the systemic hypertension were able to act without

restriction. These subcapsular JGA were, as expected, smaller than in the normal kidneys and thus comparable with the JGA found in the untouched kidney in two-kidney Goldblatt hypertension or in the primary hyperaldosteronism (Conn's Syndrome) (4, 7, 9, 12, 28).

The divergent morphological findings in different parts of the renal cortex may explain the normal PRA which occurred despite the fact that the patient was hypertensive. The elevated renin secretion of the juxamedullary JGA that were supplied by the stenosed polar artery was apparently compensated by the reduced renin secretion in the rest of the kidney cortex. This in turn means that with systemic hypertension a normal PRA is relatively too high (18-19).

Further work is required to solve the question of whether the synchronous enlargement and decrease of the Go cell field and the MD is of functional importance. A similar increase in the size of MD and the Go cell field in Bartter's syndrome and Addison's disease and a similar reduction in Conn's syndrome as in the two different types of JGA in this kidney has been reported earlier (7). In the present material there was no significant correlation between the size of the MD and its contact area with the Go cell field or the Go cell field with the arterioles. This again corresponds with previously reported results in Addison's disease and in Conn's syndrome (7).

The epithelioid transformation of the mesangial cells reported by Boldizar and Danilow (11) and by Barajas *et al.* (1) could not be confirmed in this study. In both papers the JGA were studied with the



Fig 3 JGA from the juxtamedullary cortex. Efferent arteriole at the glomerular hilus with many epithelioid cells in the media. To the left of the arteriole, some normal mesangial cells without epithelioid transformation. Afferent arteriole with numerous epithelioid cells in the media somewhat apart from glomerulus. Between both arterioles and the distal tubule with the macula densa, the epithelioid transformed Goormaghtigh cells. Plexiglass embedding, semithin section (2 μ), Giemsa stain, 570 \times

subcapsular JGA (4409 μm^2) but in neither did it differ significantly from the normal MD (5298 μm^2 Tab 2 and 3).

The Go cell field of the subcapsular JGA was also smaller than in the normal kidneys, with significantly smaller areas of direct contact with MD and the arterioles (Tab 2 and 3).

Direct contact between the MD and the arterioles was found with the afferent in two and with the efferent arteriole in four of the 11 examined juxtamedullary JGA. In the nine subcapsular JGA examined, the frequency of direct contact between MD and the arterioles was equal to that in the normal kidneys (6) i. e. it occurred 7 times with the afferent and 8 times with the efferent arterioles. No significant difference in the extent of the contact areas between the hilar arterioles and the MD was found on comparing the subcapsular JGA with the normal JGA (Tab 2 and 3).

DISCUSSION

This patient represents a natural example of the experimental Goldblatt two-kidney hypertension. The hypertension was obviously caused by the stenosed polar artery while the rest of the right and the whole left kidney had free blood flow.

It has been proved many times both experimentally and clinically that even with normal peripheral plasma renin activity (PRA) hypertension develops if one renal artery is stenosed (14, 18, 23-26, 31-33).

This constellation was present in our patient, who suffered from hypertension (Blood pressure = 170/110 mm Hg) with normal PRA values in the

TABLE 2 Area of Macula Densa Basement Membrane and the Areas of Contact between the Juxtaglomerular Structures in Juxtamedullary and Subcapsular JGA in the Affected Kidney and in Normal Reference Material (6)

	MD			Go/MD			Go/Art			MD/Art		
	\bar{x}	SD	SE	\bar{x}	SD	SE	\bar{x}	SD	SE	\bar{x}	SD	SE
Normal kidneys (n = 43)	5298 \pm 1364 (208)			2066 \pm 796 (117)			Aa 1053 \pm 462 (71)			Aa 605 \pm 387 (60)		
							Ae 584 \pm 285 (43)			Ae 266 \pm 209 (33)		
Stenosed kidney a) juxtamedullary cortex (n = 11)	5822 \pm 928 (280)			3300 \pm 662 (200)			Aa 2817 \pm 136 (409)			insufficient number with contact		
							Aa 1078 \pm 429 (129)					
							Aa 630 \pm 208 (70)			Aa 398 \pm 240 (80)		
b) subcapsular cortex (n = 9)	4409 \pm 998 (407)			1589 \pm 387 (129)			Aa 335 \pm 293 (98)			Ae 216 \pm 103 (37)		

MD: Macula densa, Go: Goormaghtigh cell field, Art: Arteriole, Aa: afferent arteriole, Ae: efferent arteriole. Mean \bar{x} with standard deviation (SD) and standard error of the mean (SE). All numbers in μ^2 .

TABLE 3. Juxtaglomerular and Subcapsular JGA in the Stenosed Kidney Compared with Each Other and with Normal Reference Material (8)

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				Aa	Ac	Aa	Ac
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electron microscope. With the light microscope we noticed a rather conspicuous difference between the epithelioid transformed Go cell in the juxtaglomerular JGA and the mesangial cells (Fig. 3).

A similar case was reported by Bergentz *et al* (2). This patient, too, had a stenosis of a polar artery with hypertension. In the peripheral blood a normal angiotensin level was found, although histological examination of the part of the kidney affected by the stenosis revealed numerous epithelioid cells in the afferent arterioles and the Go cell fields. This patient became normotensive after surgical correction of the stenosis.

A recent study in hypertensive children with segmental hypoplastic kidneys compared the renin content of kidney tissue with morphometric studies of the corresponding tissue from different parts of these kidneys. On the border between the normal and the hypoplastic parts the JGA were found to be hyperplastic, and there was a remarkable increase in renin content of the kidney tissue at the same location (16, 17).

The preoperative considerably increased total blood volume (7480 ml) in this patient was probably the consequence of a secondary aldosteronism and possibly changed tubular function in that part of the kidney which was supplied by the stenosed polar artery. Thus it is known that urine volume and sodium concentration in the urine are diminished in the stenosed kidney (26, 34, 36). Since no aldosterone-determination was made in our patient, this suggestion is supported by the fact that 9 months after operation the patient was found to be normotensive with normal PRA and normovolemic with a total blood volume of 4750 ml (Table 1).

Möhring *et al* (30) have shown an increase in blood volume following experimental stenosis of one renal artery with an intact contralateral kidney in the rat. It was not until the rats developed malignant hypertension with blood pressure higher than 180 mm Hg systolic that they lost sodium chloride and water. At the same time the already increased PRA rose very rapidly and to very high levels.

The normalized circulation and kidney function 9 months after operation clearly demonstrate that we were dealing with an early stage of hypertension, in which the functional disturbances were reversible. Moreover no secondary hypertensive changes in blood vessels or glomeruli (15, 29) had occurred in those parts of the kidneys exposed to the elevated blood pressure. Otherwise these changes might have maintained the hypertension even after its cause had been eliminated (18, 27, 30), the so-called *post-Goldblatt hypertension*.

The statistical analyses were carried out at the Institute of Electronic Data Processing, University of Bergen, Norway.

We are thankful for the perfect technical work of *Miss Alckeler* and *Miss Jørgensen*.

The photographic work was carried out with great care by *Mrs. Kopp* and *Mr. T. Christensen*.

We are very much indebted to *Mrs. Inger Sjøberg* for her excellent assistance in preparing the manuscript.

The renal angiography was performed at *St. Marco-Hospital, Lunen, Germany* Head *Dr. Gebauer*.

This study was supported by the *German Research Foundation*.

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DEMONSTRATION OF HEPATITIS B-SURFACE ANTIGEN IN LIVER BIOPSIES

*A Comparative Investigation of Immunoperoxidase and Orcein Staining on Identical
Sections of Formalin Fixed, Paraffin Embedded Tissue*

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Clasen, Per P. & Thomsen, Per. Demonstration of hepatitis B-surface antigen in liver biopsies. A comparative investigation of immunoperoxidase and orcein staining on identical sections of formalin fixed, paraffin embedded tissue. *Acta path. microbiol. scand. Sect. A*, 86: 383-388 1978.

Staining of 1000 consecutive liver biopsies with orcein showed positive reaction in the cytoplasm of ground-glass hepatocytes in 18 of the biopsies. On new sections of the 18 orcein positive biopsies immunoperoxidase staining was performed in order to demonstrate hepatitis B-surface antigen (HB_sAg). After destaining the same sections were stained with orcein. All biopsies showing positive orcein staining showed positive immunoperoxidase reaction. The number of positive cells was in biopsies with less than 20 positive cells per mm² biopsy larger using the immunoperoxidase staining than with orcein staining. Further the staining contrast was more pronounced. Immunoperoxidase staining thus seems more sensitive than orcein staining for demonstrating HB_sAg in liver tissues. Orcein stains HB_sAg in liver tissues even though the antigen determinants are blocked by antibodies.

Key words: Hepatitis B-surface antigen, liver tissue, orcein staining, immunoperoxidase staining.

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Accepted as submitted 19 in 78

Hepatitis B-surface antigen (HB_sAg) represents a group of morphologically different structures demonstrable in serum from patients with hepatitis B-virus infection as well as in serum from apparently healthy chronic carriers of hepatitis B-virus. These structures which are parts of the hepatitis B-virus have common antigen determinants (4). Morphological, histochemical and immunohistochemical investigations of liver cells have shown that the presence of HB_sAg is connected with ground-glass appearance of the cytoplasm (6), positive orcein staining (10) and positive immunofluorescence (5) or immunoperoxidase reaction (2, 7).

Previous comparative investigations between orcein staining and immunoperoxidase staining for demonstration of HB_sAg have given varying

results. Some authors (1-13) have stated that the immunoperoxidase staining was more sensitive than the orcein staining while others (8, 12) have found that the methods gave equal results. Nayak and Sachdev (9) even claim that orcein staining is the most appropriate method. This discrepancy is to some extent due to sampling error when the staining reactions are not performed on the same section. This is most pronounced in cases of small biopsies with scattered occurrence of HB_sAg. Another important cause of the discrepancy could be the variation of the orcein staining when different sources and batches of the dye are used. The purpose of the present investigation is to compare the orcein staining and the immunoperoxidase staining using the same section of formalin fixed and paraffin embedded liver tissue.

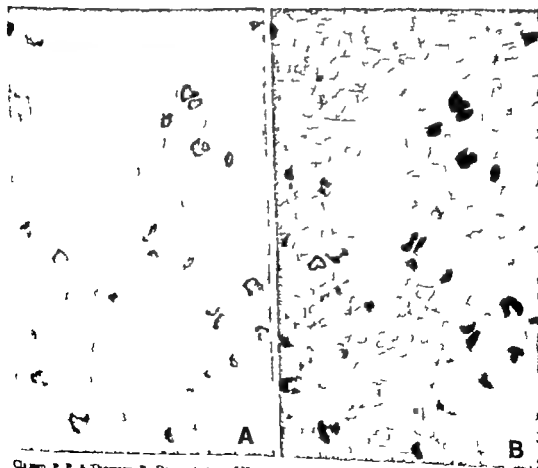
RESULTS

Eighteen out of 1000 consecutive liver biopsies showed positive orcein staining of the cytoplasm. All 18 biopsies showed positive staining for HBsAg by immunoperoxidase staining. Evaluation of the number of positive cells in the same section stained with both methods showed (Table 1) that biopsies with less than 20 positive cells per mm² biopsy showed a somewhat larger amount of positive cells using the immunoperoxidase staining than found with the orcein staining. Moreover the IP-staining showed greater contrast between the stained cells and the background (Fig. 1). With both stainings a fine granular pattern of ground-glass type was found in larger or smaller parts of the cytoplasm in the positive reacting liver cells (Fig. 2). Occasionally

TABLE 1 Results of Staining Identical Sections of Liver Biopsies for HBsAg with Orcein and by Immunoperoxidase

Number of positive cells	Number of liver biopsies	
	Orcein	Immunoperoxidase
+	4	2
++	8	10
+++	3	3
++++	3	3

Scoring system used: + 1-5 ++ 6-20, +++ 21-40
++++ > 40 positive cells per mm² liver biopsy



Clasen, P. P. & Thomsen, P. Demonstration of Hepatitis B-Surface Antigen in Liver Biopsies

Fig. 1 The same part of the same section of liver tissue stained in A with orcein and in B by the immunoperoxidase technique $\times 100$.

MATERIAL AND METHODS

The material consists of liver biopsies received at the pathological department of Hvidovre Hospital and routinely fixed in Lillie's 10 per cent buffered formalin and embedded in paraffin. Serial sections were cut on a rotary microtome at 5 μ m and placed on slides without any special treatment.

In the primary material consisting of 1000 consecutive biopsies received in the period 1/7/1976 to 30/6/1977 the routine orcein staining in 18 cases showed a positive reaction of ground-glass hepatocytes. New sections were prepared from these 18 biopsies and first stained by the immunoperoxidase technique for HBsAg and then, after destaining, stained with orcein. An additional 50 consecutive biopsies were all stained with both methods on the same section in the same manner as above.

Immunoperoxidase Technique

Sera

- Anti HBsAg serum raised in rabbits (Behringwerke-Hoechst) ORBB 14/15 batch no A 2804 F Titer RIA > 10 000 Working dilution 1 + 20 was found by titration
- Peroxidase-conjugated swine-antirabbit IgG (DAKO IG Denmark) code no P 2190 lot 017 was used in dilution 1 + 20. The conjugate has been manufactured by the two step procedure.
- Absorbed antiserum was prepared by adding 1 ml HBsAg control serum (Behringwerke-Hoechst) to 1 ml rabbit-anti HBsAg serum. The solution was left for 24 hours at 4°C and subsequently centrifuged at 5000 rpm for one hour. The supernatant was used as absorbed antiserum for the control stainings
- Normal rabbit serum (DAKO IG Denmark)

Staining Procedures

Immunoperoxidase staining was performed as an indirect technique with the following steps

- Deparaffinized sections were incubated in methanol containing 0.5 per cent H_2O_2 for 10 minutes in order to block the endogenous peroxidase activity
- Rinse in phosphate buffered saline (PBS) with 5 per cent normal swine serum (15 minutes)
- Incubation with rabbit anti-HBsAg serum diluted 1 + 20 with PBS containing 10 per cent normal swine serum (30 minutes)
- Rinse in PBS for 15 minutes
- Incubation with peroxidase conjugated swine-antirabbit IgG in dilution 1 + 20 with PBS buffer (30 minutes)
- Rinse in PBS (15 minutes)
- Incubation with 0.04 per cent 3-amino-9-ethylcarbazole and 0.01 per cent H_2O_2 in 0.05 M sodium acetate/acetic acid buffer pH 5 (15 minutes). (For 50 ml solution 20 mg 3-amino-9-ethylcarbazole dissolved in 2.5 ml N,N-dimethylformamide was used. Immediately before use 47.5 ml buffer and 25 μ l 30 per cent H_2O_2 was added).
- Rinse in tap water
- Mount in Aquamount.

Control staining was in all cases done by replacement of rabbit-anti-HBsAg serum (step 3) with absorbed antiserum and with normal rabbit serum. Control sections with known positive reaction for HBsAg was included in all staining series. In order to examine the possible influence of the peroxidase staining on its subsequent orcein staining, adjoining sections was orcein stained with and without previous immunoperoxidase staining. Further orcein staining was performed on sections previously immunoperoxidase stained following destaining and treatment for 1/2 hour with hydrochloric acid buffer pH 2 in order to break the antigen-antibody binding.

Following the immunoperoxidase staining the staining reaction was recorded semiquantitatively in the way described below and selected areas were photographed. The sections were then destained in 93 per cent ethanol for 15 minutes and subsequently stained with orcein.

Orcein Staining

Two different sources of dye and several batches from the same source have been tested in order to get the optimal contrast between the staining reaction in the liver cells and the background staining. All stainings in this material were performed with the same batch of orcein from BDH. The following modification of Shikata's method (10) was used.

Reagents

- Potassium permanganate 1.5 per cent in distilled water. Immediately before use 1.5 ml concentrated H_2SO_4 was added in 100 ml solution
- Orcein BDH batch no 2479310 1 per cent in 70 per cent ethanol was adjusted to pH 1-2 with concentrated hydrochloric acid (approx 1 ml/100 ml). The solution was usable for at least three months when kept closed at 4°C. The staining solution was stable five times within three weeks when expiration was avoided and the solution was kept at 4°C between the staining procedures)
- Oxalic acid 1.5 per cent in distilled water

Staining Procedure

200 ml coplin jars were used for 19 sections

- Sections were oxidized in the potassium permanganate solution for 5 minutes
- Destaining in oxalic acid until the sections were pale (about 1 minute)
- Staining with orcein at room temperature (4 hours)
- Dehydration in 99 per cent ethanol (2-3 minutes)
- Transferring to xylene and mounting in DPX.

After staining the selected areas of the biopsies were again photographed.

Semiquantitative Evaluation

The semiquantitative evaluation was made independently by both authors according to the following scoring system: + 1-5 ++ 6-20 +++ 21-40 ++++ > 40 positive cells per mm² liver biopsy measured at 100 times magnification.

work. Different products and different batches within the same product have provided variable results. Following standardization of the orcein staining using the same product and batch we have obtained reproducible results.

Orcein stains other structures than HB_s-Ag in liver tissues. These are elastin, ceroid and copper protein complexes. Elastin differs clearly from HB_s-Ag by the fibrillar structure and portal localization. Ceroid is found in Kupffer cells in connection with focal liver cell necrosis and has a rough granular structure differing from the diffuse fine granular structure seen in connection with HB_s-Ag. Sippow *et al.* (11) have shown that copper-protein complexes are stainable with orcein. This material is like HB_s-Ag found in the cytoplasm of liver cells, but the distinct rough granular structure has no morphological similarity to the appearance of the cytoplasm in cases with HB_s-Ag.

Previous investigations comparing the orcein staining and the immunoperoxidase staining have given differing results which, apart from the variation of orcein products, could be due to sampling error using different sections for the two stainings. We have avoided this source of error by using the same section for both stainings.

Finally different immunoperoxidase staining techniques have been used in the above mentioned papers. In one of the papers (8) the direct staining technique which generally is less sensitive than the indirect technique has been used. In all other papers (1, 9, 12, 13) indirect staining techniques have been used utilizing either peroxidase conjugated antibodies or labelled antibodies, in the latter case using peroxidase/antiperoxidase complexes (PAP). Previous unpublished investigations (3) have shown no essential difference in sensitivity between the two mentioned indirect techniques using peroxidase conjugated antibodies manufactured by the two step procedure as used in this investigation. We have chosen the indirect technique with peroxidase conjugated antibodies as it is less time consuming and cheaper than the PAP technique.

The intensity of the peroxidase staining depends on the following factors: the amount of antigen present, the antibody concentration of the used antisera, the degree of conjugation and the antibody concentration of the used conjugates. These conditions are not specified in sufficient degree in the cited papers, just as we have not been able to obtain an exact declaration on the concentration of peroxidase conjugated antibodies in the conjugate we have used. A reasonable interpretation of the differing results using varying immunoperoxidase methods is therefore not possible.

As we in earlier investigations have observed a

time dependent reduction of the activity of the peroxidase conjugates, we have included a positive control section with a known staining intensity in all staining series in order to obtain reproducible results.

Performing the immunoperoxidase staining we have used the antibodies at a dilution giving the most optimal contrast between background and positively reacting cells. Performed in this way it is obvious when comparing the stained cells in the orcein staining and immunoperoxidase staining that the staining contrast is greatest in the immunoperoxidase staining. For this reason the immunoperoxidase staining would seem to some extent to be more sensitive for the demonstration of HB_s-Ag in liver cells. A more detailed examination of the relative sensitivity of the two methods would require investigation by both methods on the same section of a larger consecutive material than presented here.

In addition our investigation has shown that it is possible to stain HB_s-Ag with orcein whether the antigen is blocked by antibodies or not. The staining of HB_s-Ag by orcein is thus hardly due to binding to the antigen determinants but more likely to other areas of the molecule.

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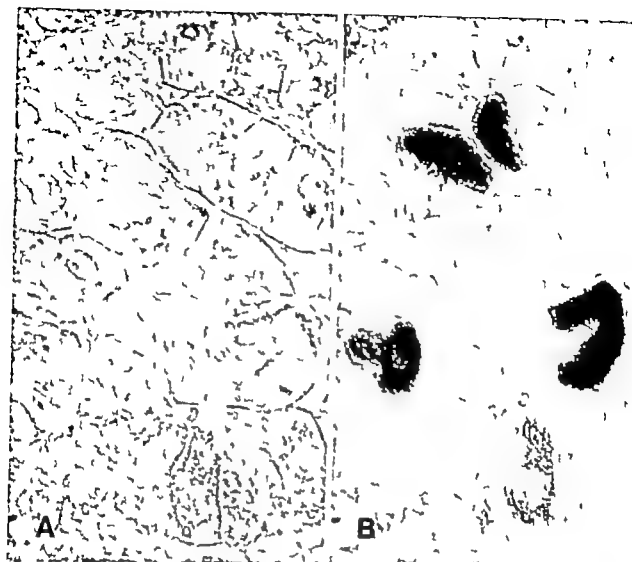


Fig. 2 Orcein staining (A) and immunoperoxidase staining (B) of liver cells shows a fine granular staining pattern of the cytoplasm by both methods $\times 400$

a vesicular pattern was found, most often in connection with fatty liver. No staining was found in the cell membrane or in the nucleus.

The distribution of positive reacting cells varied from biopsy to biopsy and within the same liver biopsy. In biopsies with only few positive cells these were distributed singly and scattered, while biopsies with more than 40 positive cells per mm^2 showed large coherent areas where all cells were positive alternating with areas where none or very few cells were positive. No special distribution of positive cells within lobules or nodules was found.

Investigation of the possible influence of the peroxidase staining and the following orcein staining showed that the number of positive reacting cells and the intensity of the staining reaction was similar whether the orcein staining was done alone or the peroxidase staining was performed on the section before the orcein staining. The same result

was obtained when after the immunoperoxidase staining the antibody HB_s-Ag binding was broken by glycylhydrochloric acid buffer before the orcein staining.

On staining 50 consecutive liver biopsies with both methods one biopsy was found showing weak positive reaction in few cells with both immunoperoxidase and orcein staining.

DISCUSSION

Our results show in agreement with previous investigations that HB_s-Ag is demonstrable by specific immunohistochemical staining in the cytoplasm of ground-glass hepatocytes showing positive orcein staining.

The histochemical mechanism of the orcein staining is still unclarified. Orcein is a mixture of more than ten different, partly unknown, compo-

TRANSTHORACIC ASPIRATION BIOPSY

Occurrence of Non-Neoplastic Cells in Biopsies from Malignant and Non-Malignant Lesions

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Frances, D. Transthoracic aspiration biopsy. Occurrence of non-neoplastic cells in biopsies from malignant and non-malignant lesions. Acta path. microbiol. scand. Sect. A, 86 389-392, 1978.

In the search for a possible distinction between false and true negative biopsies, an analysis of the distribution of the non-neoplastic cells has been performed in 100 randomized transthoracic aspiration biopsies in so many histologically verified cases. Aspiration biopsies from malignant lesions often contain many giant cells, alveolar epithelial cells, and mast cells, but a significant difference was only found for the mast cells. A negative biopsy containing many inflammatory cells and mast cells and showing a pronounced degree of necrosis may represent a chronic obstructive pneumonitis, and such a finding should always prompt further investigation.

Key words: Aspiration biopsy transthoracic.

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Received 30.1.78 Accepted 21.3.78

The majority of previous studies on diagnostic transthoracic aspiration cytology has been centered on identification and morphological appearance of tumour cells with only peripheral mention of the occurrence of non-neoplastic cells (1-5, 7).

It was our general impression that both epithelioid cells and macrophages with more than four nuclei (giant cells) were present in many non-granulomatous lesions. Further, we believed that the presence of mast cells, alveolar epithelial cells, and giant cells should be a characteristic finding in biopsies from malignant lesions.

We have therefore found it of interest in a series of transthoracic aspiration biopsies to perform an analysis of the non-neoplastic cells.

It was a hope that the results of such an analysis could be an aid in the evaluation of false negative biopsies.

MATERIAL AND METHODS

The present material comprises 100 randomized aspiration biopsies selected from the previously reported material of 227 histologically verified transthoracic aspiration biopsies (2). The 100 biopsies represent 100 patients and from each of these cases a trained cytopathologist chose the most suitable May-Grunwald-Giemsa stained smear (3).

The different non-neoplastic cells, listed in the tables were registered semiquantitatively (0, +, ++, +++, -). Regarding neutrophils, eosinophils, lymphocytes, monocytes, and thrombocytes these cells were only registered when their numbers exceeded the limits of a normal peripheral blood count, judged by the amount of erythrocytes present. Cases where registration of a certain cell type was impossible due to necrosis were marked -.

Among the non-neoplastic cells some need a further presentation. We identified mast cells by their cytoplasmic contents of dark purple granules and the epithelioid cells as macrophage-like cells with nuclei more than

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TABLE 2. Distribution of Non-Neoplastic Cells in Group I a (71 Transbronchoscopic Aspiration Biopsies Containing Malignant Tumour Cells) versus Group II b (14 Biopsies from Histologically Non-Neoplastic Lesions)

	Group I a					Group II b					Mann-Whitney	Difference (chi ² -test)
	0	++	-	0	++	++	-					
Erythrocytes	3	17	13	36	2	0	3	3	6	2		
Neutrophils	11	29	17	10	4	5	4	1	2	2		$p < 0.05$ (la > Ib)
Eosinophils	44	9	0	0	18	11	0	0	1	2		$p < 0.05$ (la < Ib)
Lymphocytes	18	26	9	4	14	7	5	0	0	2		
Monocytes	42	0	0	0	29	12	0	0	0	2		
Thrombocytes	32	8	1	0	30	4	3	1	3	3		$p < 0.05$ (la < Ib)
Plasma cells	33	16	2	1	19	8	2	1	0	3		$p < 0.05$ (la > Ib)
Mast cells	18	19	9	13	12	8	4	0	0	2		$p < 0.05$ (la > Ib)
Macrophages with one nucleus	4	27	12	25	3	3	5	1	3			$p < 0.05$ (la > Ib)
Macrophages with 2-4 nuclei	22	26	11	5	7	5	4		1	2		
Macrophages with > 4 nuclei	33	23	2	4	9	9	3	0	0	2		
Endothelial cells	30	21	3	0	17	8	1	1	2	2		$p < 0.05$ (la < Ib)
Cholangio-coloncar epithelial cells	43	11	1	1	15	7	1	1	1	4		$p < 0.05$ (la < Ib)
Non-cholangio-coloncar epithelial cells	45	11	0	0	15	7	3	0	0	4		
Normal alveolar epithelial cells	76	22	7	5	11	5	3	3	0	3		
Abnormal alveolar epithelial cells	54	4	1	2	8	8	2	1	0	3		
Metastatic cells	46	12	2	1	10	9	1	1	0	3		
Degree of necrosis	7	30	17	17		6	5	2	1		$p < 0.05$ (la > Ib)	$p < 0.01$ (la > Ib)
presence/absence (+++ ++ +/0)												
large numbers/small numbers (+++ ++/+ 0)												

TABLE 3. Distribution of Non-Neoplastic Cells in Transbronchoscopic Aspiration Biopsies, Group I b (10 False Negative Biopsies) versus Group II b (14 Biopsies from Histologically Non-Neoplastic Lesions)

	Group I b					Group II b				
	0	+	++	+++	-	0	+	++	+++	-
Erythrocytes	0	3	3	3	1	0	3	3	6	2
Neutrophils	4	2	2	1	1	5	4	1	2	2
Eosinophils	8	1	0	0	1	11	0	0	1	2
Lymphocytes	5	2	2	0	1	7	5	0	0	2
Monocytes	8	1	0	0	1	12	0	0	0	2
Thrombocytes	6	0	0	3	1	4	3	1	3	3
Plasma cells	9	0	0	0	1	8	2	1	0	3
Mast cells	5	1	0	3	1	8	4	0	0	2
Macrophages with one nucleus	1	5	1	2	1	3	5	1	3	2
Macrophages with 2-4 nuclei	4	4	0	1	1	5	4	2	1	2
Macrophages with > 4 nuclei	6	2	0	1	1	9	3	0	0	2
Endothelial cells	5	3	0	1	1	8	1	1	2	2
Cholangio-coloncar epithelial cells	6	1	1	1	1	7	1	1	1	4
Non-cholangio-coloncar epithelial cells	5	3	1	0	1	7	3	0	0	4
Normal alveolar epithelial cells	1	2	4	2	1	5	3	3	0	3
Abnormal alveolar epithelial cells	6	1	0	2	1	8	2	1	0	3
Metastatic cells	0	7	2	1	1	9	1	1	0	3
Degree of necrosis						6	5	2	1	

No significant differences between the groups were revealed.

twice as long as wide. The presence of red coloured cilia was used to distinguish between the two types of columnar epithelial cells. Normal alveolar epithelial cells are in this work defined as rounded to polyhedral cells with a diameter of about 12-20 μ m, a nuclear cytoplasmic ratio of about $\frac{1}{2}$ and light finely vacuolated cytoplasm only seldom containing granules or dust. These cells could be solitary but more often occurred as small monolayered sheets of 3-8 cells. Atypical alveolar epithelial cells were distinguished from the last mentioned cells by their nuclei being more condensed and more than twice the size of the normal. Mesothelial cells appeared as polyhedral cells in smaller or larger monolayered sheets, the cytoplasm being bluish, occasionally with a few small vacuoles, the nuclei uniform and medium stained.

Based on the final histological findings there were 81 cases of malignant intrathoracic tumours and five cases of benign tumours. In seven cases granulomatous lesions were found, and the remaining cases comprised the following lesions: localized fibrosis (three cases), abscess (two cases), pulmonary infarction (one case), and pneumocystis carinii (one case).

The material was divided into two groups: Group I comprising 81 aspiration biopsies from histologically malignant lesions, and Group II comprising aspiration biopsies from histologically non-malignant lesions. The cytological features of the non-neoplastic elements of the

two groups have been compared. For evaluation of a possible difference in the distribution of the non-neoplastic cells in the false negative biopsies and the true negative biopsies, the biopsy groups were subdivided.

Group I was divided into:

- I a. 71 biopsies containing reproducible malignant tumour cells
- I b. 10 biopsies without reproducible malignant tumour cells.

Group II was divided into:

- II a. 5 biopsies from histologically benign tumours
- II b. 14 biopsies from histologically non-neoplastic lesions.

A comparison was made between subgroups I a and II b and subgroups I b and II b.

For the statistical assessment the Mann-Whitney's non-parametrical test has been used for the comparison of the semiquantitative distribution of the non-neoplastic cells, the chi²-test for the comparison of the qualitative distribution (presence/absence (+++ ++ +/0) or large numbers/small numbers (+++ ++ + 0) of the non-neoplastic cells. The limits for type I-error (2 α) have been set at 0.05.

TABLE 1. Distribution of Non-Neoplastic Cells in Group I (81 Trans thoracic Aspiration Biopsies from Histologically Malignant Lesions) versus Group II (19 Biopsies from Histologically Benign Lesions)

	Group I					Group II					Mann-Whitney	Difference (chi ² -test)
	0	+	++	+++	-	0	+	++	+++	-		
Erythrocytes	3	20	16	39	3	0	4	3	10	2		
Neutrophils	15	31	19	11	5	7	7	1	2	2		
Eosinophils	52	10	0	0	19	15	0	1	1	2	$p < 0.05$ (I < II)	$p < 0.05$ (I > II)
Lymphocytes	23	28	11	4	15	10	7	0	0	2	$p < 0.05$ (I > II)	
Monocytes	50	1	0	0	30	17	0	0	0	2		
Thrombocytes	38	8	1	3	31	9	3	1	3	3		
Plasma cells	42	16	2	1	20	13	2	1	0	3	$p < 0.05$ (I > II)	$p < 0.05$ (I > II)
Mast cells	23	20	9	16	13	9	7	0	1	2		
Macrophages with one nucleus	5	32	13	27	4	4	7	1	5	2		$p < 0.05$ (I > II)
Macrophages with 2-4 nuclei	26	30	11	6	8	8	5	1	1	2		
Macrophages with > 4 nuclei	19	25	2	5	10	13	3	1	0	2		
Epitheloid cells	35	24	3	1	18	10	3	2	2	2	$p < 0.05$ (I < II)	
Columnar epithelial cells	49	12	2	2	16	10	1	2	2	4	$p < 0.05$ (I < II)	
Non-columnar epithelial cells	50	14	1	0	16	10	4	0	1	4		
Normal alveolar epithelial cells	27	24	11	7	12	8	3	4	1	3		
Abnormal alveolar epithelial cells	57	6	3	3	12	12	2	2	0	3		
Mesothelial cells	52	13	2	3	11	14	1	1	0	3		
Degrees of necrosis	14	30	19	18		11	6	2	1		$p < 0.01$ (I > II)	$p < 0.05$ (I > II)

presence/absence (+++ ++ +/0)
large numbers/small numbers (+++ ++ + 0)

TRANSTHORACIC ASPIRATION BIOPSY

The Distribution of the Non-Neoplastic Cells in Aspiration Biopsies from Different Types of Malignant Lung Tumours

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FRANCE D Transthoracic aspiration biopsy. The distribution of the non-neoplastic cells in aspiration biopsies from different types of malignant lung tumours. *Acta path. microbiol. scand. Sect. A, 86 393-395 1978*

In an earlier paper concerning classification of aspirated May-Grunwald-Giemsa stained malignant tumour cells from lung lesions, we found the sensitivity to be 80-95 per cent for adenocarcinomas, undifferentiated carcinomas and small cell carcinomas. For epidermoid carcinomas the sensitivity was only 64 per cent. It was therefore found of interest to see if a registration of the non-neoplastic cells could be an aid in the cytological tumour cell classification. An analysis has been performed of the distribution of the non-neoplastic cells in 71 aspiration biopsies from six different types of malignant lung tumours. A variegated population of many and different types of inflammatory cells, mast cells and thicker epithelial cells was a characteristic finding in biopsies from neoplasms with an epidermoid differentiation.

Key words: Aspiration biopsy, transthoracic.

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Received 30.1.78 Accepted 21.3.78

Preoperative classification of malignant lung lesions is of increasing importance for the institution of the correct specific treatment. Correct classification is likewise important when deciding whether a tumour is metastatic or of primary origin. (1,5)

In an earlier paper we have shown an agreement of 75 per cent in the cytological classification performed on transthoracic aspiration biopsies in a histologically classified material. (2) Whereas the sensitivity of the cytological classification was found to be high in biopsies from adenocarcinomas, undifferentiated carcinomas and small cell carcinomas (80-95 per cent), it was rather low in biopsies from epidermoid carcinomas (64 per cent).

It is possible that the occurrence of non-neoplastic cells may differ in biopsies from different types of malignant tumours and thereby be an aid in the cytological classification.

For these reasons a comparison has been made

between the occurrence of non-neoplastic cells in transthoracic aspiration biopsies from different types of histologically classified malignant lung tumours.

MATERIAL AND METHODS

The material consists of 71 transthoracic aspiration biopsies containing reproducible malignant tumour cells (3). All the biopsies were included in the previously reported material of 100 randomized transthoracic aspiration biopsies (3, 4).

The aspiration biopsies were grouped according to histological tumour types (6). The non-neoplastic cells were registered semiquantitatively (0 + + + + +) according to the analysis described in the previous report (4).

Twenty-six biopsies represented epidermoid carcinomas. Of these seven biopsies were in the original cytological report as well in the ultra- and microobservation study (2, 3) classified as undifferentiated carcinomas. This group of seven biopsies was compared to the

RESULTS

The semiquantitative distribution of the non neoplastic cells in the biopsy groups is given in Tables 1, 2 and 3 together with information about significant differences found by the Mann Whitney a non-parametrical test and the χ^2 -test.

The statistical evaluation of the registered parameters has as described in Methods included a comparison between presence/absence (+++ ++ +/0) and large numbers/small numbers (+++ ++/+ 0). The choice of the latter mode of collocation is due to the fact that it is easy to make this distinction.

DISCUSSION

Macrophages with more than four nuclei (giant cells) are in this series equally often seen in biopsies from malignant and benign lesions and not mainly in non neoplastic lesions, half of which are granulomatous. This is due to their presence in large numbers in epidermoid carcinomas (4). Epithelioid cells, though appearing more often in biopsies from non-neoplastic lesions, are equally often found in false and true negative biopsies.

The content of inflammatory cells corresponds to the degree of necrosis which is more frequent in biopsies from malignant lesions and this is so even in group of false negative biopsies. The presence of many lymphocytes in an aspirate may be due to chronic inflammation or may represent the contents of hilar lymph nodes. It should be noted that extreme numbers of lymphocytes may point to a lymphoma. (6)

Biopsies from malignant lesions often contain giant cells, alveolar epithelial cells and mast cells, but a significant difference is only found for the mast cells though not valid with regard to the group of false negative biopsies.

It is assumed that the combination of many

different inflammatory and reactive cells, especially many alveolar epithelial cells, giant cells, and mast cells found in a pulmonary aspirate is explained by the chronic obstructive pneumonitis which so often is found in connection with malignant growth (4).

The analysis has shown certain differences in the distribution of the non-neoplastic cells in primary aspirates from malignant and non-malignant lesions. No non neoplastic cell type, alone or in combination with others, can, however by its presence even in large numbers indicate anything about malignancy in an aspirate not containing malignant tumour cells.

The occurrence, however of many inflammatory cells, mast cells, and a pronounced degree of necrosis speaks in favour of an obstructive pneumonitis. Such findings must always prompt further investigation.

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the tumour groups. Most pronounced, however, is the difference seen for biopsies from epidermoid carcinomas versus each of the other groups. Any significant difference of this kind is indicated in the table.

In Table 2 the non-neoplastic cellular distribution in the groups of incorrectly and correctly classified epidermoid carcinomas, and for comparison also the group of undifferentiated carcinomas is given. In addition the significant differences found between these biopsy groups are given.

DISCUSSION

As seen from the results of the non-neoplastic cell analysis the aspirate from each tumour type shows a rather characteristic cell picture.

Epidermoid carcinomas appear with a variegated population of many and different non-neoplastic cells indicating severe reactive inflammation and necrosis. Characteristic is an occurrence of many macrophages with more than four nuclei (giant cells), mast cells, and alveolar epithelial cells. This may partly be explained by the growth-pattern of this tumour. Epidermoid carcinomas frequently cause an occlusion of the affected bronchus leading to formation of chronic obstructive pneumonitis (8). In addition it may be assumed that direct contact between keratinized epidermoid tumour cells and stroma cells produce a severe irritative stromal reaction. This could be paralleled to the reaction often seen in simple epidermoid cysts with inflammation (7).

Adenocarcinomas only show a slight degree of necrosis and inflammatory reaction. The growth pattern in lung adenocarcinomas seems to differ from the epidermoid carcinomas by propagating along the air passages to a large extent and bronchial obstructions are more seldom seen. Moreover in contradistinction to the isolated keratinized epidermoid tumour cells, tumour cells from adenocarcinomas, even formations of tumour cells producing considerable amounts of secretion, as in colloid carcinomas, are often seen without signs of surrounding stromal reaction.

Undifferentiated carcinomas and small cell carcinomas show a moderate degree of necrosis and inflammatory reaction.

Macroepidermoid carcinomas not surprisingly

due to their epidermoid component, appear with a non-neoplastic cell population and necrosis similar to epidermoid carcinomas.

Sarcomas. Our biopsies show very sparse necrosis and inflammatory reaction, explained by the fact that two of the three biopsies represent pleural and not pulmonary tumour growth.

By separating the group of incorrectly classified epidermoid carcinomas and comparing these with both the group of correctly classified epidermoid carcinomas and the group of undifferentiated carcinomas only a few significant differences are found. The contents, however, of many inflammatory cells, giant cells, alveolar epithelial cells, and mast cells in an aspirate showing undifferentiated epithelial tumour cells may point towards the tumour being epidermoid in origin, and hereby lead to an intensified search for tumour cells showing signs of keratinisation.

To this very limited extent the recording of the pattern of non-neoplastic cells may be of some diagnostic use, in spite of the fact that the classification of malignant tumours must, of course, always be based on the cytomorphological appearance of the tumour cells themselves.

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group of 19 correctly classified epidermoid carcinomas and to the group of 10 undifferentiated carcinomas.

The Mann Whitney's non-parametrical test was employed. The limit for type I-error (2 α) was set at 0.05

RESULTS

Table 1 shows the distribution of the non-neoplastic cells in the six histological types of lung tumour. Differences are seen for several cell types between

TABLE 1 *Distribution of Non-Neoplastic Cells in 71 Aspiration Biopsies from Different Types of Malignant Lung Tumours*

	Epidermoid carcinomas					Small cell carcinomas					Adeno-carcinomas					Undifferentiated carcinomas					Macroadenoid carcinomas					Squamous Metastases				
	0	+	++	+	-	0	+	+	-	0	+	+	-	0	+	+	0	+	+	0	+	++	0	+	++					
Erythrocytes	1	2	4	17	2	1	3	2	4	0	1	5	2	10	0	0	5	4	1	0	0	0	1	3	0	0	2	0	1	1
Neutrophils	1	8	9	4	4	4	4	1	1	0	3	12	2	1	0	3	4	3	2	0	0	1	1	2	0	2	0	1	0	1
Eosinophils	13	2	0	0	11	7	2	0	0	1	12	3	0	0	3	7	1	0	0	2	2	1	0	0	1	3	0	0	0	0
Lymphocytes	1	12	2	1	10	3	2	3	2	0	7	5	3	0	3	2	5	1	1	1	2	2	0	0	0	3	0	0	0	0
Monocytes	12	0	0	0	14	6	0	0	0	4	12	0	0	0	6	7	0	0	0	3	2	0	0	0	2	3	0	0	0	0
Thrombocytes	9	1	1	0	15	3	3	0	0	4	10	2	0	0	6	6	1	0	0	3	2	0	0	0	2	1	0	0	0	0
Plasma cells	8	5	1	1	11	5	3	0	0	2	12	2	0	0	4	3	5	1	0	1	2	1	0	0	1	3	0	0	0	0
Mast cells	2	4	5	8	7	5	3	0	1	1	4	7	3	2	2	4	2	1	1	2*	1	2	0	1	0	2	1	0	0	0
Macrophages with one nucleus	0	10	4	10	2	0	4	1	4	1	0	8	4	6	0	2	2	3	3	0	0	2	0	0	2	0	2	1	0	0
Macrophages with 2-4 nuclei	5	8	3	4	4	3	2	2	1	2	5	12	1	0	0	4	3	2	0	1	2	1	1	0	0	0	0	0	0	0
Macrophages with > 4 nuclei	4	13	2	3	4	6	1	0	1	2*	12	5	0	0	1	3	3	0	0	2	3	1	0	0	0	0	0	0	0	0
Epithelioid cells	9	8	2	0	7	5	2	0	0	3	6	8	0	0	4	4	3	1	0	2	3	0	0	0	1	3	0	0	0	0
Ciliated columnar epithelial cells	11	5	1	0	9	7	0	0	0	3	11	4	0	1	2	8	1	0	0	1	3	1	0	0	0	0	0	0	0	0
Non-ciliated -- epithelial cells	14	3	0	0	9	7	0	0	0	3	10	6	0	0	2	8	1	0	0	1	3	1	0	0	0	0	0	0	0	0
Normal alveolar epithelial cells	3	8	4	4	7	4	2	0	0	2	8	7	1	1	1	7	2	0	0	1	2	2	0	0	0	0	0	0	0	0
Abnormal alveolar epithelial cells	18	4	0	0	4	9	0	1	0	0	15	0	0	2	1	8	0	0	0	2*	3	0	0	0	1	3	0	0	0	0
Mesothelial cells	9	10	0	1	6	8	0	0	0	2	16	1	0	0	1	6	1	2	0	1	4	0	0	0	0	0	0	0	0	0
Degree of necrosis	1	9	7	9	0	1	3	3	3	0	2	11	3	2	0	2	5	1	2	0	0	0	3	1	0	1	2	0	0	0

This parameter shows a significantly lower value than in epidermoid carcinomas (Mann-Whitney's non-parametrical test $p < 0.05$).

TABLE 2 *Distribution of Non-Neoplastic Cells in Transthoracic Aspiration Biopsies from 7 Incorrectly Classified Epidermoid Carcinomas 19 Correctly Classified Epidermoid Carcinomas and 10 Undifferentiated Carcinomas*

	Incorrect					Correct					Undifferentiated				
	0	+	++	+++	-	0	+	++	+++	-	0	+	++	+++	-
Erythrocytes	0	1	0	5	1	1	1	4	12	1	0	5	4	1	0
Neutrophils	1	4	1	0	1	0	4	8	4	3	1	4	3	2	0
Eosinophils	4	0	0	0	3	9	2	0	0	8	7	1	0	0	2
Lymphocytes	1	2	1	0	3	0	10	1	1	7	2	5	1	1	1
Monocytes	3	0	0	0	4	9	0	0	0	10	7	0	0	0	3
Thrombocytes	2	0	1	0	4	7	1	0	0	11	6	1	0	0	3
Plasma cells	2	0	1	0	4	6	5	0	1	7	3	5	1	0	1
Mast cells	0	2	2	2	1	0	8	2	8	1	2	2	3	3	2
Macrophages with one nucleus	1	2	1	1	2	4	6	4	3	2	4	3	2	0	1
Macrophages with 2-4 nuclei	0	5	0	0	2	4	8	2	3	2	5	3	0	0	2
Macrophages with > 4 nuclei	1	2	1	0	3	8	6	1	0	4	4	1	1	0	2
Epithelioid cells	3	0	0	0	4	8	5	1	0	5	8	1	0	0	1
Ciliated columnar epithelial cells	3	0	0	0	4	11	3	0	0	5	8	1	0	0	1
Non-ciliated -- epithelial cells	0	1	0	2	4	3	7	4	2	3	7	2	0	0	1
Normal alveolar epithelial cells	3	1	0	0	3	15	3	0	0	1	8	0	0	0	2
Abnormal alveolar epithelial cells	1	1	0	1	4	8	9	0	0	2	6	1	2	0	1
Mesothelial cells	1	1	1	4	0	0	8	6	5	0	2	5	1	2	0
Degree of necrosis															

fewer neutrophils than in the correctly classified epidermoid carcinomas (Mann-Whitney's non-parametrical test $p < 0.05$)

more normal alveolar epithelial cells than in the undifferentiated carcinomas (Mann-Whitney's non-parametrical test $p < 0.05$)

TRANSTHORACIC ASPIRATION BIOPSY

The Occurrence and Significance of Alveolar Epithelial Cells

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FRANCIS, D. Transthoracic aspiration biopsy. The occurrence and significance of alveolar epithelial cells. *Acta path. microbiol. scand. Sect. A*, 86: 397-400, 1978.

Alveolar epithelial cells in pulmonary aspirates sometimes appear with a pronounced nuclear atypism which may give rise to diagnostic difficulties. An analysis has been performed of the occurrence and morphology of alveolar epithelial cells in a series of histologically verified transthoracic aspiration biopsies not containing clear-cut malignant tumour cells. Alveolar epithelial cells, as well as atypism of these cells, are more often seen in biopsies from histologically malignant cases than in benign cases. Their presence in a pulmonary aspirate may be due to a chronic obstruction atelectasis. In cases with tumour-like configuration of the alveolar epithelial cells, an open pulmonary biopsy is recommended, as it seems impossible to decide whether such findings represent a neoplastic or a reactive process.

Key words: Aspiration biopsy, transthoracic, alveolar epithelial cells.

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Accepted as submitted 7 iv 78

In two previous reports (4, 5) concerning an analysis of the non-neoplastic cells in pulmonary aspirates, alveolar epithelial cells were registered in many biopsies from both malignant and non-malignant lesions. In some biopsies these cells appeared with a pronounced nuclear polymorphism, the diagnostic significance of which was unknown to us.

Alveolar epithelial cells, pneumocyte type I (6, 7), are well-known constituents of pulmonary aspirates (2, 8) but, as far as we know, no analysis has been performed of the distribution and morphology of these cells in different types of pulmonary lesions.

The following investigation was made in order to find out whether the occurrence of the alveolar epithelial cells and the cytological appearance might be of any diagnostic help in the evaluation of aspirates from different intra-thoracic lesions.

MATERIAL AND METHODS

The primary material consists of 227 histologically verified transthoracic aspiration biopsies (3). As the occurrence of obvious malignant tumour cells may bias the analysis of atypical alveolar epithelial cells, all biopsies with one or more slides containing clear-cut tumour cells from histologically verified malignant lesions were excluded (139 cases). Of the remaining 88 cases, specimens were available in 80 cases; these cases are the material of the present study.

On the basis of the final histological findings there were 36 cases of malignant intrathoracic tumours, 10 cases of benign tumours and 34 cases of non-neoplastic lesions.

The 80 biopsies comprise a total of 452 slides. Fourteen are wet-fixed slides which are Papanicolaou stained, whereas the rest are air-dried and stained by the May-Grunwald-Giemsa method.

Cytological investigation. The slides were mixed and then screened for alveolar epithelial cells (see Fig. 1A).

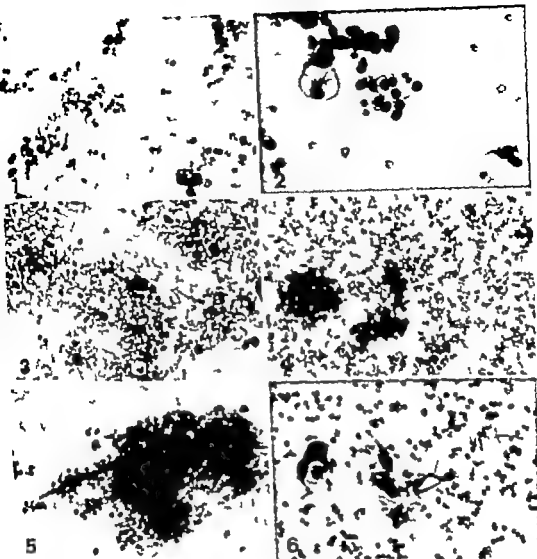


Fig 1 Primary excrete showing normal alveolar epithelial cells (sec) appearing as single cells or in small sheets. There are also erythrocytes and macrophages containing variable amounts of granules. (MGG $\times 250$).

Fig 2 Same as Fig 1. Centrally sheet of normal sec. Macrophages in the upper left corner, one of which is vacuolated. (MGG $\times 400$).

Fig 3 Centrally group of atypical sec, surrounded by macrophages. In the left part of the picture, several normal sec. (MGG $\times 200$).

Fig 4 Centrally a group of small, closely-packed sec in a gland-like formation. Also two groups of macrophages. Patient with adenocarcinoma. (Case 1) (MGG $\times 250$).

Fig 5 Large group of tumour-like sec. To the left, the sec are arranged along capillaries in gland-like structures. Patient with recurrent adenoma. (Case 2) (MGG $\times 250$).

Fig 6 Large sec with tumour-like configuration. The cells are small-nucleated with pronounced nucleoli. Patient with relapsed papilloma. (Case 3) (MGG $\times 250$).

quantitative registration (0 + ++) was performed. Slides without preserved non neoplastic pulmonary cells were registered (-) as non-representative material.

Furthermore, it was noted whether the aec appeared to be normal, showed evidence of nuclear polymorphism (some nuclei being twice as large as normal) or were arranged in a tumour-like configuration (clumping or grouping of cells resembling gland-like structures)

These registered data were now rearranged in groups corresponding to the individual cases. A synopsis of each case was made, taking on the one hand the most pronounced occurrence and on the other hand the most pronounced atypism. Each of these parameters was then correlated to the histological findings.

For the statistical evaluation, the χ^2 -test and Fisher's exact test were used. The limit for type I error (2 α) was set at 0.05

RESULTS

Normal aec (Fig. 1 and 2) are rounded to polyhedral cells with diameter of about 12–20 μ m, a nuclear cytoplasmic ratio of about $\frac{1}{2}$ and light, finely vacuolated cytoplasm only seldom containing granules or dust. These cells may be solitary but more often occur as small mono-layered sheets of 3–8 cells.

Atypical aec (Fig. 3) have more condensed nuclei that are more than twice the normal size. These cells may be solitary or may occur in smaller or larger mono-layered sheets.

Aec with tumour like configuration (Fig. 4 5 and 6) show nuclear polymorphism, and some of the cells are grouped in clusters or arranged in gland-like structures (Fig. 5).

As shown in Table 1 aec are seen in about $\frac{1}{4}$ of the representative biopsies, and they appear more often in biopsies from malignant lesions (27 out of 29) than in benign lesions (23 out of 33) ($p < 0.05$)

Table 2 shows that atypical aec are registered in both the malignant and the non-neoplastic group, and they occur more often in the malignant group (13 out of 26) than in the non-malignant group (3 out of 21) ($p < 0.05$).

Tumour-like appearance of the aec is seen in 3 cases, one from each histological group.

In one case, represented by Fig. 4 the histological sections showed scar-tissue adenocarcinoma with pronounced epithelialization of the alveoli and a gradual transformation from these cells into veritable tumour cells.

The second case was an 18 year-old girl. She had a benign tumour resected from her left lung two years previously. The histological diagnosis was pulmonary adenoma. The aspiration biopsy from the tumour at the site of the resection showed numerous tumour cells of the alveolar epithelial cell type (Fig. 5). The nuclei were polymorphous and the cells were grouped in clusters. A recurrence of the tumour was diagnosed, and this was in accordance

TABLE 1 *Semi-quantitative Registration of Alveolar Epithelial Cells (aec) in Aspiration Biopsies from Histological Groups*

Alveolar Epithelial Cells	Histological Groups		
	Malignant Tumours	Benign Tumours	Non-Neoplastic Lesions
Many aec (++)	19	4	11
Few aec (+)	8	3	5
No aec (0)	2	3	7
Non Representative material (-)	7	—	11

TABLE 2 *Occurrence of Normal and Atypical Alveolar Epithelial Cells (aec) in Aspiration Biopsies from Histological Groups*

Alveolar Epithelial Cells	Histological Groups		
	Malignant Tumours	Benign Tumours	Non-Neoplastic Lesions
Tumour like Configuration of aec	1	1	1
Atypical aec	13	—	3
Normal aec	13	6	12

COMPARISON OF ANOXIC CHANGES IN ISOLATED RAT CARDIAC MYOCYTES IN SUSPENSION AND IN HISTOLOGICAL SECTIONS

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Rajs, J, Jones, D. P & Jakobsson, S W Comparison of anoxic changes in isolated rat cardiac myocytes in suspension and in histological sections. Acta path. microbiol. scand. Sect. A, 86 401-408, 1978

Cardiac myocytes from adult rat were isolated by heart perfusion in the presence of collagenase and incubated in the absence and presence of oxygen. As a result of anoxia, there was a gradual increase in plasma membrane permeability noted as an increase in succinate-stimulated oxygen uptake, a decrease in trypan blue exclusion frequency, a leakage of cytosolic lactate dehydrogenase activity and an increased proportion of swollen, irregularly contracting myocytes. The contractions of the damaged myocytes resembled the known non-physiologic contractions of the heart, i.e. extrasystoles, arrhythmia, fibrillation or block. After different periods of time of anoxia myocyte pellets were fixed in formalin, sectioned and examined by light microscopy. The appearances of the myocytes in suspension were compared with those in paraffin-embedded sections. Special attention was given to the hematoxylin basic fasten picro acid stain and it was noted that the basic fasten was taken up by contracted or damaged myocytes, which according to their morphology in suspension revealed irregular contractions, but not by undamaged or necrotic myocytes.

Key words: Rat cardiac myocytes, light microscopy, anoxia, suspension, membrane permeability, histological sections.

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Received 25 vi 78 Accepted 25 vii 78

Morphological diagnosis of pre-necrotic myocardial lesions of local ischemic or general hypoxic origin in tissue sections is so far an unelucidated problem. Conventional appearances in early stages of myocardial ischemia, such as eosinophilia and fragmentation of the myofibres or interstitial hemorrhages and oedema have been reported to be non-specific, not sufficiently convincing and influenced by early autolytic changes (Proceedings from the first meeting of ISNOD 1976). Uptake of basic and acid fasten in the myofibres, their waviness and appearance of zonal lesions or irregular contraction bands have been considered as more reliable signs of early myocardial damage (Liu *et al* 1971; Foley *et al* 1964; Bouchardy & Majno 1971; Martin *et al* 1969); in our experience, however their interpretation is still open to question (Rajs & Jakobsson 1976).

In our laboratory a method has been developed for the isolation of beating cardiac myocytes from adult rats with the aim of studying the function and morphology of these structures (Rajs *et al* 1978). The purpose of the present investigation was to compare anoxic changes in isolated, suspended beating rat myocytes observed in the light microscope with the appearances and staining properties of rat myocytes in paraffin-embedded sections, with special reference to experiences from routine post mortem myocardial sections.

*Anoxia is used here to refer to the condition of complete oxygen deprivation which we have attempted to create. Experimentally the cells are exposed to less than 1% of the O₂ required for maximal respiration (see Material and Methods).

with the histological findings of the reoperation specimen

The third case has been mentioned several times in our previous reports. It happened to be our only false-positive biopsy diagnosis. This 39 year-old woman had had a mastectomy followed by radiation treatment. A lung infiltrate behind the scar was punctured and the aspirate showed tumour-like cells. (Fig 6) The surgical biopsy however showed radiation pneumonitis.

Atypism and/or tumour-like configuration of the aec was found more often in biopsies from malignant lesions (14 out of 27) than in non-malignant lesions (5 out of 23).

DISCUSSION

In this study alveolar epithelial cells (aec) are often seen in biopsies from malignant and benign lesions, and their presence is therefore no indication of malignancy. The occurrence of aec, however, does clearly indicate that the aspiration has been made, at least partly from pulmonary tissue.

The occurrence of aec in large numbers and especially in combination with inflammatory cells of different types, suggests an inflammatory reaction as in chronic obstructive pneumonitis, which is so often found in connection with malignant growth (4, 5) but the mere occurrence of large numbers of aec does not imply malignancy.

The occurrence of atypical aec may give rise to false-positive diagnostic results, but as the cytological polymorphism is only slight, experience will prevent this error. On the other hand, this study shows that atypical aec occur more often in false-negative biopsies (biopsies not containing clear-cut malignant tumour cells from histologically malignant tumours) than in true-negative biopsies.

The occurrence of atypical aec should therefore always prompt further investigation.

Aec arranged in a tumour-like configuration seldom occur. An open surgical biopsy is recommended in such cases, as it seems impossible to decide whether these cells represent a neoplastic or a reactive process (1, 2).

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RESULTS

Oxygen uptake, NADH penetration and trypan blue exclusion frequency of isolated rat cardiac myocytes incubated in the presence or absence of oxygen are shown in Table 1. Stimulation of oxygen uptake by exogenously added succinate was maximal after 30 minutes of anoxia whereas the rate of NADH penetration into the cells was optimal after 45 minutes of incubation with N_2 as the gas phase. In contrast, when incubation was performed in the

presence of carbogen gas there was little loss in cell viability during the 90 minutes observation period.

Our observations of the vital properties of the myocytes in suspension and the staining properties of the myocytes in paraffin-embedded sections are presented in Table 2. Light microscopic examination of those myocytes, which excluded trypan blue, revealed for the most part rod-shaped cells showing striations and nuclei but no vacuoles (Fig. 2). About one third of the myocytes which excluded trypan blue were more or less irregular in shape; they were

TABLE 2. Comparison of Myocyte Morphology, Contractility and Staining Properties when Stained with Trypan Blue in Suspension with the Appearance of the Myocytes in Paraffin Sections Stained with Hematoxylin-Eosin and Hematoxylin Basic Fuchsin Picric Acid Stains in Subsequent Phases of Oxygen Deprivation

	General appearance of the myocytes	Myocytes in suspension		Myocytes in paraffin sections	
		Contractility	Trypan blue stain	Hematoxylin-eosin stain	Hematoxylin basic fuchsin picric acid stain
0	»Rod-shaped« Striated	Rhythmic, varying in frequency	Exclusion.	No alterations.	No uptake of basic fuchsin.
x					
y	Shortened and thickened (contracted) Striated	Tachyarrhythmic. »Extrasystoles«		Shortened, like with fragmentation in tissue sections	Slight, patchy uptake of basic fuchsin
e					
e	Increasing in size. Single subsarcolemmal and extracellular blebs Striated	Arrhythmic. »Extrasystoles«.		Eosinophilic	Patchy diffuse uptake of basic fuchsin.
a					
d	Swollen, but still rectangular with increasing number of blebs Irregular thick striations	Arrhythmic. Fibrillation			Uptake in the irregular transverse bands and in the terminal portions.
e					
p	Extremely swollen, with outballoing subterminal segment Numerous blebs Striations, seen only in terminal portions or patchily Bursting follows	Isolated areas with arrhythmic contractions Block between the terminal portions	Slight, patchy uptake	Hyalineization.	Intense »crimson-red« stain. Maximal uptake of basic fuchsin.
h					
i	Immediately after the bursting No striations. Increasing number of extracellular blebs	No contractions, but some isolated areas with jerks	Intense blue stain.		Slight patchy uptake of basic fuchsin.
l					
o	Pycnotic, surrounded by numerous blebs	No jerks		Necrosis	Picrinophilic
a					

MATERIAL AND METHODS

Beating cardiac myocytes from adult rats were obtained as previously described (Rajs *et al* 1978). A suspension of myocytes in Ca^{2+} free Krebs-Henseleit buffer was mixed with an equal part of 0.33% trypan blue in the same buffer. Individual cells were observed in suspension for up to 3 hours in the light microscope at room temperature, without oxygen supply with respect to alterations in morphology, contractility and trypan blue staining properties. The integrity of the plasma membrane was evaluated by determining oxygen consumption in the absence and presence of succinate and NADH penetration into the cells when NADH and pyruvate were added to the medium. (Orrenius *et al* 1976; Högberg & Kristoferson 1977). Oxygen concentration was measured with a Clark electrode calibrated relative to air-saturated distilled H_2O . O_2 concentration in the perfusion medium was about 0.75 mM and during heart cell incubation in the presence of carbogen was estimated to be 0.4–0.6 mM. O_2 concentration in cell incubations exposed to N_2 was below 10^{-6} M and was difficult to estimate accurately. The total O_2 content of the N_2 gas and the gas flow rate (assuming 100% O_2 transfer from the gas to the aqueous phase) was 1.0 nmol/min/ml which was less than 1% of that consumed at the maximum respiration rate.

A suspension containing about $45 \pm 3 \times 10^6$ heart muscle cells (10^6 cells/ml), about 50% of which excluded trypan blue (Fig. 1), was divided in two equal parts. Part one was bubbled with carbogen gas (95% O_2 and 5% CO_2) at 37°C . Part two was immediately after the harvest bubbled with N_2 . Samples from the two incubates were observed in the microscope after 15, 30, 45, 60, 75 and 90 minutes of exposure to carbogen gas or N_2 . The remainder was centrifuged at 37 g for 90 sec in 15 ml glass tubes. The supernatant was discarded and the pellet resuspended in a 4% neutral formaldehyde solution and recentrifuged as above. After 24 to 48 hours of fixation these pellets were embedded in paraffin. Sections, 3–5 μm thick, were cut out with a microtome knife and stained with hematoxylin-eosin (HE) and Mallory's phosphotungstic acid hematoxylin (PTAH) as



Fig. 1 Suspension of isolated cardiac myocytes from adult rat showing regularly wood-shaped and deformed but not stained myocytes as well as deformed blue stained myocytes. Cells were suspended with 0.33% trypan blue and observed with lowered condenser. Magnification of the diapositive $\times 50$.

routine staining methods, and von Kossa's method to demonstrate intracellular calcium (Armed Forces Institute of Pathology 1960). Acid fuchsin (Poley *et al* 1944) and hematoxylin basic fuchsin picric acid (HBF) stainings (Lle *et al* 1971) were employed to demonstrate preneoplastic lesions. The staining technique and mode of interpretation of the results when using the last method were performed in accordance to our previous experience (Rajs & Jakobsson 1976). Since in the pellets of isolated myocytes no erythrocytes, elastic or collagen fibers were present for comparison of uptake of the basic fuchsin, various differentiation times were applied (5, 10, 20, 30, 45, 60 and 75 sec) and slides with the most distinct carmalum-red color were utilized for interpretation of morphological alterations. Myocytes which were stained too dark red to distinguish the structure were further differentiated in solution C.

All chemicals were standard commercial products. The results are presented as typical experiments of which three or more were performed with separate batches of cells.

TABLE 1 Oxygen Uptake, NADH Penetration and Trypan Blue Exclusion Frequency in Isolated Cardiac Myocytes Incubated in the Presence and Absence of Oxygen

Incubation time, minutes	Oxygen uptake, nmol/min/ 10^6 cells				NADH % of maximal penetration		Trypan blue exclusion, % of total	
	no addition		+ succinate %					
	O_2	N_2	O_2	N_2	O_2	N_2	O_2	N_2
0	61.0	61.0	3.9	3.9	2	2	42	42
15	61.1	36.1	3.2	17.1	7	52	46	24
30	63.7	21.2	3.1	30.5	8	90	36	15
45	62.6	11.1	3.9	26.0	7	100	23	9
90	50.9	8.0	4.4	12.5	10	100	25	2

% stimulation of O_2 uptake upon addition of Na-succinate

RESULTS

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	General appearance of the myocytes	Myocytes in suspension		Myocytes in paraffin sections	
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2	Shortened and thickened (contracted). Striated	Tachyarrhythmic »Extrasystoles.		Shortened, like with fragmentation in tissue sections	Slight, patchy uptake of basic fuchsin.
3	Increasing in size. Single subterminal and extracellular blebs	Arrhythmic »Extrasystoles		Eosinophilic	Patchy diffuse uptake of basic fuchsin.
4					
5	Se often, but still rectangular with increasing number of blebs. Irregular thick striations	Arrhythmic Fibrillation			Uptake in the irregular transverse bands and in the terminal portions.
6					
7	Extremely swollen, with outbulging nodular segment. Numerous blebs	Isolated areas with arrhythmic contractions. Block between the terminal portions	Slight, patchy uptake.	Hyalinization	Intense »crimson-rose stain. Maximal uptake of basic fuchsin.
8	Striations, seen only in terminal portions or patchily. Bursting follows				
9					
10	Immediately after the bursting. No striations. Increasing number of extracellular blebs	No contractions, but some isolated areas with jerks.	Intense blue stain.		Slight patchy uptake of basic fuchsin.
11					
12	Pycnotic, surrounded by numerous blebs	No jerks.		Necrosis	Picrinophilic

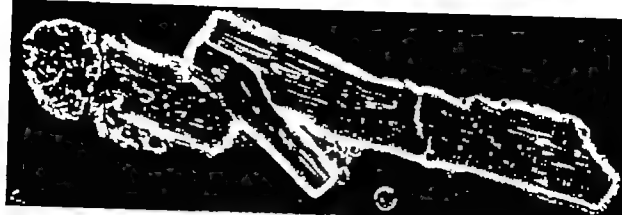


Fig 2 Photomicrograph of five myocytes in suspension. Two on the right which are connected with intercalated disk contracted rhythmically with about 30 beats/minute. The third from the right revealed tachyarrhythmia in the upper end. The fourth from the right was contracted and showed about 10 irregular beats/minute. The round pycnotic myocyte on the left showed no contractions and was stained with trypan blue. Phase contrast microscopy X 390

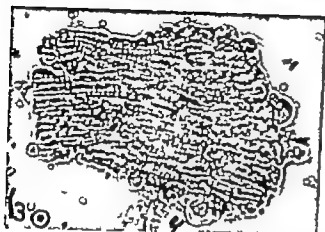


Fig 3 Photomicrograph of a cardiac myocyte in suspension short time before bursting. It is contracted and swollen, reveals vacuoles and irregular striations. There are parts with extracellular blebs and indistinct plasma membrane. The right terminal portion was in fibrillation, while the left one revealed bradyarrhythmia

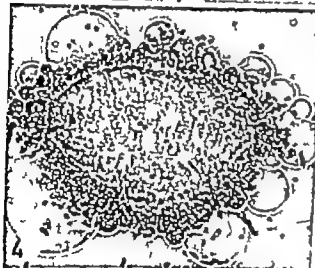


Fig 4 The same myocyte after bursting. The myocyte had ceased to contract, and one can see only traces of striations. Note many extrasarcoplasmic granules and blebs, which were continually growing



Fig 5 The same myocyte about 1 hour after bursting. It is pycnotic and dark, due to trypan blue penetration. Lowered condensor X 403 (Figs. 3, 4 and 5)

shortened and swollen in the central part and sometimes spheric (Fig. 3). These cells also showed intrasarcoplasmic, often subsarcolemmal located vacuoles, and irregularity of the cross striations as well as surface blebs which increased in size with time of observation. The contents of these blebs appeared to be granular and/or gaseous.

Rhythmic contractions of the myocytes - up to 60 beats per minute - were noted. In our experiments, all rod-shaped myocytes made contractions at one time or another if the observation time was only long enough. In some instances, only one contraction per several minutes was recorded. With time, the contractions became gradually more frequent and the rhythm less regular. Some myocytes displayed premature contractions, of extrasystolic character independent of the cell rhythm. With an increase in the beating frequency

the irregularity of the rhythm they sometimes give the impression of cell fibrillation. Later a very irregular contraction rhythm was noted, still later the myocytes exhibited patchy often focally located areas with autonomous contractions or fibrillations. The terminal portions of the myocytes divided by swollen medial segments, also had an uncoordinated rhythm. Sometimes, a contraction wave would pass from one caudal portion of the myocyte through the subterminal zone of the swollen medial segment opposite terminal portion. On this way altered myocytes could show some trypan blue stained nuclei in their sarcoplasm. Quite suddenly such swollen myocytes would burst, the contractions would disappear and the myocytes cease to contract (Fig. 7) Subsequently the plasma membrane became roiled and indistinct and the surface blebs nume-

rous and large, exceeding with their total size the myocyte itself. Such an altered myocyte did not exclude trypan blue and usually stained instantly (Fig. 5). However even after uptake of trypan blue stain, the myocytes continued to shrink while the blebs increased sometimes displaying single jerks within some part of the cell. Thus, a single rod-shaped myocyte could gradually undergo all these described structural stages and types of contractions. The time required for these alterations to take place varied between some twenty or thirty minutes and three to four hours.

After 15 minutes of oxygen exposure, the number of myocytes in suspension which excluded trypan blue, usually increased (Table 1), the rod-shaped cells became more elongated and the contractions more recurrent. In contrast, the myocyte suspension exposed to N_2 for 30 minutes

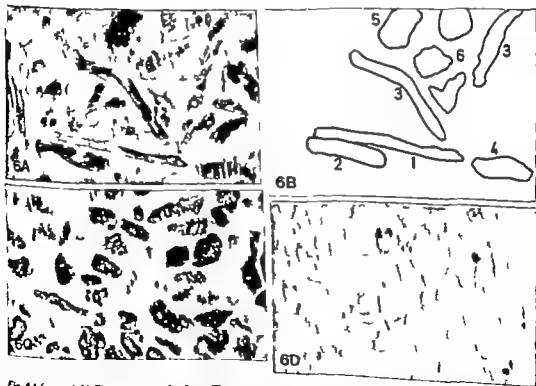


Fig. 6A (upper left) Photomicrograph of paraffin-embedded section of rat cardiac myocytes, fixed immediately after isolation. B (upper right) stands for interpretation of appearances in A: 1 - rod-shaped, undamaged, perinucleolar myocyte, 2 and 3 - some less deformed or contracted myocytes with slight or intense uptake of basic fuchsin (red stain), 4 - contracted myocyte revealing red stained contraction bands, 5 - extremely swollen, deformed myocyte, 6 - three necrotic myocytes. C (lower left) Myocytes fixed after 30 minutes oxygen deprivation. Most of them show massive uptake of basic fuchsin within contraction bands or diffusely. D (lower right) Necrotic myocytes after 60 minutes of oxygen deprivation. Perinucleolar is seen after only 10 sec of differentiation. Differentiation time for A) and C) was 30 sec. HBFP stain. Magnification of exposure X 200.

revealed microscopically only few rod-shaped myocytes, the majority of the cells being more or less altered, as described above. No rod-shaped myocytes were seen after 60 minutes of N_2 exposure.

Light microscopic examination of sections of paraffin-embedded myocyte pellets showed, when stained with HE and PTAH stains, that the appearance of the undamaged myocytes was quite comparable to the conventional morphology of myocytes in tissue sections, while the altered myocytes were shortened, swollen or rounded, showing homogeneous blebs. The sarcoplasm of the damaged myocytes was eosinophilic and more or less homogenous and hyalinized. Uptake of acid fuchsin was noted neither in intact, nor in damaged myocytes. Sections stained according to von Kossa showed no intracellular calcium even after one hour of oxygen deprivation. Sections that were fixed and embedded immediately after the harvest of the myocytes, and stained according to the HBFP technique, showed most distinct «crimson red» stained myocytes when differentiated for 30 and 45 seconds in solution C the stain intensity could however vary among simultaneously stained sections of the same myocyte pellet. Shorter processing time resulted usually in overstaining of the myocytes with basic fuchsin, while too long differentiation in solution C resulted in their decolorization. When optimally differentiated, the rod-shaped heart myocytes had become yellowish i.e. picrinophilic and revealed only rarely a minimal uptake of basic fuchsin in perinuclear areas and/or in the terminal portions, the latter appearances reminding of zonal lesions. Some shortened, swollen myocytes showed irregular thickened and red stained contraction bands. Totally deformed swollen, or rounded myocytes revealed either homogeneous, hyalinized, «crimson red» stained sarcoplasm and yellowish pericellular blebs, or were quite picrinophilic. The necrotic cell detritus was picrinophilic (Figs. 6A and B).

HBFP staining of the embedded myocyte suspension, exposed to N_2 during 15 minutes, revealed an appearance similar to what is described above, only very few rod shaped cells were noted. These cells decreased in number even more after 30 minutes of exposure to N_2 when the general appearance of the myocytes became rather monotonous, more or less swollen and rounded-up cells with «crimson-red» stained, thick, irregular striations, or homogenous hyalinized red stained sarcoplasm (Fig. 6C). After 60 minutes of exposure of the myocytes in suspension exposed to N_2 the HBFP-stained sections revealed no rod-shaped myocytes, but only some swollen, picrinophilic, or red stained cells in the picrinophilic, amorphous detritus (Fig. 6D).

DISCUSSION

Isolated beating cardiac myocytes from adult rats have been recommended as an experimental tool for biochemical and ultrastructural studies (Velosky *et al.* 1969 Gould & Powell 1974 Farmer *et al.* 1977). However light microscopic observations on isolated myocytes in suspension have up to now mostly been restricted to evaluation of the viability of the myocytes and the quality of the suspension for further biochemical studies. The myocytes have been described with regard to their contractile shape (rod-shaped, contracted, and rounded-up) and vital staining properties when stained with trypan blue. The spheric appearance of the myocyte which lost its morphological details was termed «contracture» (Bloom 1970).

The conversion of the rhythmically contracting rod-shaped trypan blue-resistant myocyte to an amorphous, non-contracting, blue stained mass was in our experiments spontaneous and slow and culminated in the burst of the swollen cell. The cause of this conversion could not always be clearly defined, since it sometimes started already during the heart perfusion. However this process could be triggered or accelerated when the suspension of myocytes was incubated with N_2 , i.e. exposed to oxygen deprivation (cf Table 1). On the other hand, an increasing percentage of trypan blue exclusion and elongation of rod-shaped myocytes after 15 minutes of oxygenation indicated some recovery of damaged myocytes. The elucidation of the process of cytopathogenic transformation might provide a better understanding of the static morphology of the paraffin-embedded tissue sections and be helpful in further electron microscope studies.

The HE, PTAH and acid fuchsin staining methods applied to the sections of paraffin-embedded myocytic pellets after various intervals of incubation with N_2 gave, in our hands, no noteworthy information. The appearance of some cellular forms – not previously observed in tissue sections – such as spheric myocytes surrounded with blebs – was interpreted as an effect of cellular swelling in the absence of supporting connective stroma. Myocytes with comparable lesions appear probably much less deformed in tissue sections, although the cross striations may be destroyed. Von Kossa's method to demonstrate calcium was negative, although calcium granules were observed in mitochondria of dog cardiac myocytes already after 40 minutes of ischemia followed by 2 minutes of reflow (Klöner *et al.* 1974). However HBFP staining resulted in various degrees of uptake of basic fuchsin in the damaged myocytes, while the undamaged and the necrotic myocytes were picrinophilic, a finding which is in good agreement with

the observations of *Lee et al.* (1971). The morphologic appearance demonstrable with this technique was often very convincing, especially when uptake of the stain was correlated with the cellular alterations and located to specific regions and structures of the myocytes. The occurrence of staining in damaged but not in undamaged or necrotic cells suggests that changes in cytosolic components must occur prior to staining and that cell death results in loss of these staining components. Succinate stimulation of respiration also appears to have this pattern (Table 1) with damaged cells (30 min) showing greater stimulation than undamaged or necrotic cells (90 min). Perhaps this stimulation can only occur when the permeability of succinate into the cell is increased by membranal changes, but when the cells undergo autolysis, ADP is lost and the respiration is no longer stimulated by succinate.

Intracellular vacuoles were described as an early sign of myocytic injury and were related to the dilated elements of the transverse tubular system, when the plasma membrane is still intact (*Berry et al.* 1970). *Csapo et al.* (1972) described, in their electron microscopic studies of the effects of large doses of isoproterenol on the rat heart, already 6 minutes after the administration of the drug, a presence of myofibrillar fragmentation, contraction band formation, and hyalinization associated with dilation of the sarcoplasmic reticulum. *Kloer et al.* (1974) observed contraction bands, extremely swollen cells, formation of vacuoles and large subnuclear blebs, which appeared capable of compressing adjacent capillaries in papillary muscles of dogs, following 40 minutes of circumferential artery occlusion and 2 minutes of blood reflow. They attributed these appearances to a defect in cell volume regulation during the phase of ischemia. These phenomena were also noted in our experiments, although the light microscope did not help in elucidating the fine structures.

The contraction bands as well as the zonal lesions are believed to be reversible (*Martin et al.* 1969). Myocytes with such lesions contract arrhythmically in suspension and exclude trypan blue, but in paraffin sections readily take up basic fuchsin. Therefore, many of the myocytes which are stained with basic fuchsin in these sections may be only reversibly injured. Immediately before bursting, when they were already deformed, with disorganized areas of contractions, the myocytes in the suspension started to absorb trypan blue and were intensely red stained or hyalinized in HBFP stained sections. Plasma membrane injuries, also established in biochemical assays (cf Table 1), and a continuous leakage of granular substance were

apparent when such cells were observed in the light microscope. These granules, which never took up basic fuchsin when stained with HBFP seemed in our preliminary electron microscopic examinations to be conglomerates of mitochondria. The swelling of the cell could be the result of an influx of Na^+ and H_2O (*Whalen et al.* 1974) followed by bursting of the plasma membrane; the latter may be considered the moment of cell death. This period is probably preceded by a period of irreversible cell injury which in our experiments could agree with the period when the myocyte is severely deformed and fibrillating and when the trypan blue and HBFP stainings overlap. Only after cell death were the myocytes completely stained by trypan blue. Thus the exclusion of this stain cannot be taken to indicate a lack of cellular damage, only that the cell is not dead. All the alterations, leakage, and signs of activity afterwards could therefore be regarded as supravital reactions.

Observations of contractility of rat heart myocytes in suspension showed that a single isolated myocyte can reveal similar contractile characters as the heart as a whole. An isolated, noninjured heart myocyte contracts autonomously but with large variations in contraction frequency. An isolated, but damaged myocyte contracts pathologically. Tachyarrhythmia, ventricular fibrillation, bradyarrhythmia, arrhythmia, fibrillation, autonomous contractility of different parts of the myocyte, as well as conduction disturbances and even block between the terminal portions of a single myocyte were noted. This behavior has to the best of our knowledge, never been recorded and suggests that the damaged myocytes, in post mortem tissue sections might have before death contracted pathologically.

Financial support was obtained from *Karolinska Institute*. The authors are indebted to Professor *Sven Öberg* for support and valuable advice, and to Mrs *Monica Sundberg*, Mrs *Gun-Britt Sundberg* and Mr *Sven Thorsell* for technical assistance.

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STRUCTURAL CHANGES IN KIDNEYS OF PATIENTS WITH OLIGURIC EXTRACAPILLARY GLOMERULONEPHRITIS DURING IMMUNOSUPPRESSIVE THERAPY

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Faarup P, Nørgaard T, Elling F & Jensen H. Structural changes in kidneys of patients with oliguric extracapillary glomerulonephritis during immunosuppressive therapy. *Acta path. microbiol. scand. Sect. A*, 86: 409-414, 1978.

Structural changes in kidney biopsies were investigated from five patients with primary oliguric extracapillary glomerulonephritis in whom the renal function was adequately maintained during extended combined immunosuppressive treatment. The most important structural change was a pronounced decrease in the number of crescents. Reduction in numbers of crescents in the late biopsies was significantly greater than the increase in the number of hyalinized glomeruli. Tubular atrophy was limited and showed only slight diffuse atrophy and a moderate interstitial fibrosis was always present during the latter stages of treatment. Disappearance of crescents in the glomeruli was not accompanied by disappearance of immunoglobulins. Successful immunosuppressive treatment of extracapillary glomerulonephritis causes the disappearance of structural characteristics of the kidney that are diagnostic for this disease.

Key words: Glomerulonephritis, crescent nephritis, renal failure, immunosuppression.

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Received 5.11.78 Accepted 16.11.78

The spontaneous clinical course of extracapillary glomerulonephritis is characterized by rapidly occurring, irreversible uremia (Brown *et al.* 1958, Harrison *et al.* 1964). In some patients with extracapillary glomerulonephritis an oliguric onset will be found. A predominant histological feature of the disease is a formation of crescents in more than 50 per cent of the glomeruli. Crescent formation is accompanied by acute glomerular alterations as focal necrosis, fibrin deposits and infiltration with polymorphonuclear leucocytes.

In primary oliguric extracapillary glomerulonephritis a combined immunosuppressive treatment consisting of azathioprine and steroid administra-

tion may maintain an adequate renal function for years (Jensen *et al.* 1974, Møller & Jørgensen 1975, Cameron *et al.* 1975, Kirkaldy-Smith 1975). Since the immunosuppressive treatment markedly changes the clinical course of this disease, the histological alterations during treatment might give information of the basis for such a fundamental prognostic improvement.

In the present work structural changes in the kidneys from five patients with oliguric extracapillary glomerulonephritis were examined. Renal function was adequately maintained by immunosuppressive treatment. Histological comparisons were made of initial lesions and histological changes during the latter stages of therapy.

PATIENTS AND METHODS

Patients. Two male and three female patients, age 32-61 years, suffering from oliguric extracapillary glomerulonephritis were treated with prednisone and azathioprine with a beneficial effect upon the renal function (*Jensen et al 1974*) (Table 1). Renal biopsy was performed initially and 12-50 months later. Immunosuppressive treatment was administered during the entire period.

Pathology. Kidney biopsies were Carnoy fixed and the paraffin embedded sections were stained with HE, van Gieson Hansen, PAS and silver methenamine. The number of vascularised glomeruli, glomeruli with cellular and/or sclerotic crescents and sclerosed glomeruli was separately counted. In addition the following lesions were noted: glomerular necrosis and/or fibrin deposits, glomerular haemorrhages, glomerular hypercellularity, presence of microthrombosis and monolayered hypercellularity of the epithelial cells inside Bowman's capsule.

Immunofluorescence microscopy was performed on the primary biopsy from patients A and C and the second biopsy from all cases. Frozen sections were incubated with FITC-marked rabbit anti-human globulin A, G, M and anticomplement C₃. Controls with non-labelled test-specimens were also performed.

RESULTS

Light microscopy. Diagnosis of primary organ extracapillary glomerulonephritis was based upon criteria described by *Jensen et al. (1974)*. The most important histological parameter was that the number of glomeruli with crescents exceeded 50 per cent.

In the five cases investigated the number of cellular crescents in the late biopsies (*i.e.* after 9-50 months of chemotherapy) was consistently reduced.

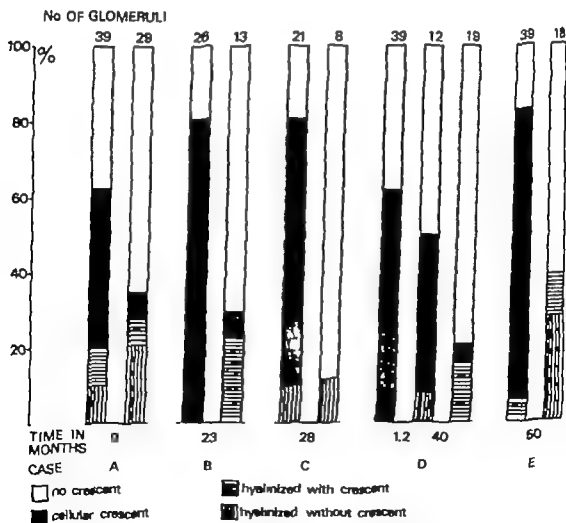


Fig 1 The frequency of glomerular crescents in per cent in initial and late biopsies. The pronounced reduction in cellular crescents during treatment is obvious in all 5 cases. Among the hyalinized glomeruli those possessing a hyalinized crescent are indicated separately in the figure. The increase in hyalinized glomeruli in the late biopsies is clearly inferior to the reduction in crescents. Possibly caused by the interstitial fibrosis present, the late biopsies were always smaller in size than the corresponding initial specimens.

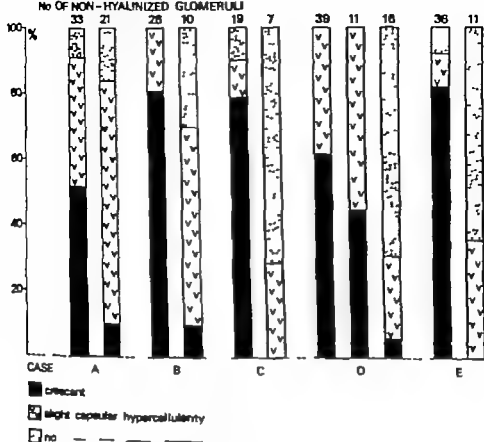


Fig. 2 In the non-hyalinized glomeruli of the initial and late biopsies the crescent occurrence as well as the presence of slight monolayered capsular hypercellularity are indicated. The disappearance of crescent in the nephrons was sometimes accompanied by the persistence of a slight hypercellularity of cells lining Bowman's capsule.

to less than 20 per cent of the values observed in initial biopsies. During the observation period the number of hyalinized glomeruli was slightly increased, but this increase was significantly less than the reduction in the number of cellular crescents (Figs. 1 and 2). The most pronounced changes were found in case C and E in whom no cellular crescents were present 28–50 months after the initial biopsy. Histological changes were independent of the interval between the first and the last biopsy; the sampling period varied from 9 to 50 months. It should be noted however that a treatment period of five weeks could not be found to induce any significant decrease in the number of crescents (Case D Figs. 1 and 2). Although cellular crescents were found in the late biopsies, they were invariably smaller and often exhibited small, hyalinized areas contrasting to those of the initial biopsies. In

addition, in late biopsies small, totally hyalinized crescents could be found, but the glomeruli appeared only slightly altered (Fig. 6). These changes were regarded as crescent scars from initial cellular crescents. In nephrons in which no crescents were found in the late biopsy a slight capsular hypercellularity one cell layer thick was frequently noted (Fig. 5). Localized glomerular or crescent necrosis, hemorrhage and presence of polymorphs in the glomeruli were frequently observed in initial biopsies, but these cellular changes were never seen in late biopsies.

In the late biopsies the tubules were generally unaffected or only slightly atrophic in nephrons exhibiting insignificant glomerular changes. In both cortical and medullary tubules the presence of casts and of necrotic cells in the tubular lumen was clearly decreased in the late biopsies. In addition, the

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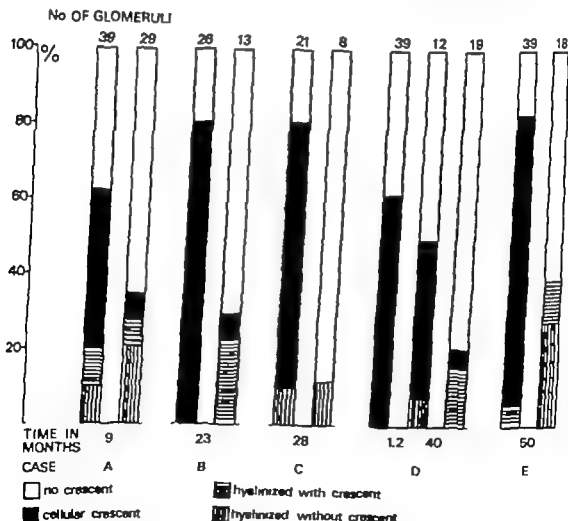


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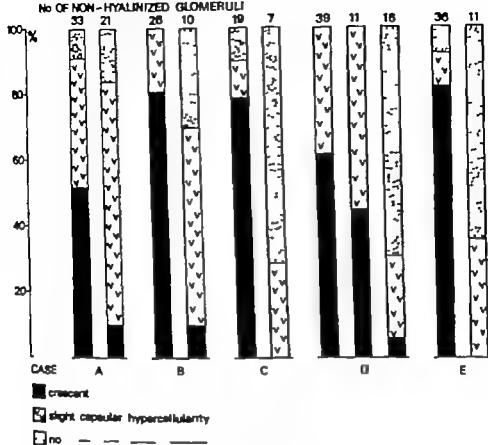


Fig 7 In the non-hyalinized glomeruli of the initial and late biopsies the crescent occurrence as well as the presence of slight monolayered capsular hypercellularity are indicated. The disappearance of crescent in the nephrons was sometimes accompanied by the persistence of a slight hypercellularity of cells lining Bowman's capsule.

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Fig 3 Case D initial biopsy Abundant crescent formation with compression of the glomerular tuft and obliteration of the lumen of the proximal convoluted tubule where it arises from the glomerulus (arrow). (Silver impregnation, $\times 260$).



Fig 4 Case D late biopsy 40 months after initial biopsy In the mesangium focal thickening and slight hypercellularity is present. Otherwise the glomerulus is essentially undamaged. Also the surrounding tubules are well preserved (Silver impregnation, $\times 320$).

TABLE I Renal Function and Immunopathology

Case	Sex	Age at initial biopsy	Biopsy no	Time in months from initial biopsy	Creatinine clearance ml/min	Immune deposits glomeruli			
						IgA	IgG	IgM	C ₃
A	F	49	I	9	19	0	+++	0	+++
			II		42	0	+++	0	+++
B	F	61	I	23	<1		not done		
			II		22	0	0	+	++
C	F	49	I	28	30	0	0	0	0
			II		51	0	0	0	0
D	M	42	I	12	51		not done		
			II		74		not done		
			III		81	+	0	+	+
E	M	52	I	30	69		not done		
			II		42	0	++	0	++

Linear immune deposits

+ Faint

++ Moderate

+++ Strong



Fig 5 Case A, late biopsy 9 months after initial biopsy. The glomerulus is hypercellular and proliferation of the cells in Bowman's capsule is present as frequently observed in the late biopsies. Fibrotic changes are present in the capsule (arrows). Tubular atrophy and interstitial fibrosis are present, these changes being typical for the late biopsies. (H & E, $\times 210$)



Fig 6 Case C, late biopsy 28 months after initial biopsy. In the glomerulus an adhesion to Bowman's capsule is present corresponding to a solidified area of the tuft (arrows). Interstitial fibrosis and focal tubular atrophy are found, these changes being typical for the late biopsies (Silver impregnation, $\times 320$). Inset: The glomerular tuft was sometimes found focally adherent to the hyalinized crescent in late biopsies. Case D 3rd biopsy (Silver impregnation, $\times 210$)

initial interstitial edema and cellular infiltration was routinely replaced by a slight to moderate interstitial fibrosis.

In the arteries and arterioles the frequency of focal PAS-positive hyalinization of the vascular wall was slightly increased in the late biopsies, but no severe vascular lesions were present. Acute necrotic changes of the vascular wall were not observed in initial or late biopsies.

Immunofluorescence microscopy Immunofluorescence microscopy was carried out on the initial biopsy of two of the five cases and on the late biopsies from all cases (Table 1). Immune deposits were present in the glomeruli from four of the five patients. In one case (case C) both the initial and the late biopsy were found to be immunologically negative. In one of the four cases (case A) with

immune deposits in the glomeruli both the initial and the late biopsy revealed linear pattern along the basement membranes of the glomeruli (Table 1).

DISCUSSION

The purpose of the present work was to evaluate the structural changes in kidneys from patients with extracapillary glomerulonephritis showing an acute, oliguric onset, in whom a combined immunosuppressive treatment maintained adequate renal function up to several years. In untreated patients the course of disease is almost always that of irreversible uremia (Heptinstall 1974). In the five patients investigated the clinical course distinctly differs from the quite persistent appearance of irreversible uremia among untreated patients.

The most striking histological observation in the

biopsies from the treated patients was the constant, significant reduction in the number of glomerular crescents in the late biopsies (Figs. 1 and 2). Furthermore, localized glomerular and crescent necrosis, infiltration with polymorphs and focal hemorrhages frequently observed in initial biopsies were never encountered in the late biopsies. In one case (D) no significant reduction in the number of crescent was found in the second biopsy taken five weeks after the initial biopsy but a third biopsy of the patient taken 40 months after treatment onset demonstrated the significant crescent decrease found in all patients. Histopathological changes observed during immunosuppressive treatment is the most likely explanation for the radical change in the clinical course of the extracapillary glomerulonephritis (Jensen *et al.* 1974). Similarly a tendency towards disappearance of cellular crescents and of necrotizing glomerulitis was noticed in the late biopsies from 3 patients, who were treated by a regimen of intensive plasma-exchange, steroids and cytotoxic drugs (Lockwood *et al.* 1977).

The few glomerular microthromboses observed in the initial as well as in the late biopsies did apparently not cause impairment of renal function during the observation period. This is in agreement with Cameron *et al.* (1975) but does not coincide with observations made by Kinkaid-Smith *et al.* (1968) who described an improvement in renal function during anticoagulation therapy in patients with extracapillary glomerulonephritis.

Parallel with the observed reduction in glomerular crescents in the late biopsies the number of hyalinized glomeruli was somewhat increased in all five cases investigated. However the increase in these non functioning nephrons was lower than the decrease observed in the number of glomeruli with cellular crescents. The treatment appears to induce a complete disappearance of some of the cellular crescents. In four of the five cases the deposition of immunoglobulins could be observed in the late biopsies, the only exception being case C, in whom immunoglobulins were equally absent in the initial biopsy. This observation indicates, that the combined immunosuppressive treatment is unable to eliminate the deposition of immunoglobulins in the glomeruli in extracapillary glomerulonephritis (Table 1). However it should be noticed, that

deterioration in renal function was reported in a patient with extracapillary glomerulonephritis caused by the intermission of the cytostatic treatment being administered for 18 months (Cameron *et al.* 1976).

In conclusion, the use of a combined immunosuppressive therapy which radically changed the otherwise rapidly progressive course of extracapillary glomerulonephritis was found to include significant systematic renal structural changes, the most important being the disappearance of cellular crescents in most glomeruli.

We wish to thank Dr C. Bruu, Ph. D., who supplied us with slides from one of the initial biopsies in this study.

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ULTRASTRUCTURE AND ACTIN DISTRIBUTION IN NEOPLASTIC NEUROGENIC CELLS IN CULTURE

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Haugen, Å. & Lærum, O. D. Ultrastructure and actin distribution in neoplastic neurogenic cells in culture. *Acta path. microbiol. scand. Sect. A*, 86: 415-426, 1978

The ultrastructure of 13 neoplastic cell culture lines derived from BD IX-rat brain was investigated in relation to growth pattern, the degree of aneuploidy and tumorigenicity. Neoplastic transformation was induced by transplacental administration of ethylnitrosourea (ENU). The neoplastic cell culture lines were either obtained from solid neuroepithelial tumours (V-lines), or developed in culture after transfer of fetal rat brain cells shortly after exposure to ENU *in vivo* (BT-lines). By transmission electron microscopy the following ultrastructural characteristics were found in both types of neoplastic cell lines: varying numbers of microtubules, few or no microfilament bundles and a variable amount of 10 nm (thin) filaments, as well as atypical nuclear structure. High numbers of 10 nm filaments were found in strongly aneuploid lines. By immunofluorescence with anti-serum against actin, the paucity of microfilament bundles was also visualized. In most cell lines a diffuse cytoplasmic actin fluorescence was found, but some tumours actin-containing bundles appeared as irregular streaks. For comparison, secondary cells from untreated fetal rat brains were also investigated. They showed parallel actin bundles throughout the cytoplasm, but these disappeared when the cells were induced to differentiate to an astrocyte-like morphology by addition of a protein fraction from adult brains. A diffuse positive reaction over the whole cytoplasm was then seen, much in the same way as in neoplastic cells.

Key words: Gliomas, meningiomas, transmission electron microscopy, cell culture, cytoskeleton.

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Received 22 iv 78 Accepted 20 iv 78

In a recent study we investigated surface properties of neoplastic cell lines in culture derived from the rat central nervous system (Haugen & Lærum 1978a). We observed an apparent relationship between surface activity as evidenced by the presence of multiple microvilli, filopodia, ruffling membranes and relictic blebs on one side and a number of biological properties of the cells on the other. Thus, high surface activity seemed to be connected with the degree of aneuploidy and tumorigenicity. It also appeared that the surface activity was highest in cell cultures giving rise to glioma-like tumours upon reimplantation into syngeneic hosts, and low in those giving rise to neuroblastoma-like tumours (with one exception).

We here report on studies of the internal

ultrastructure of the cells of these cultures. Particular emphasis has been given to the cytoskeletal system, since this may be of critical importance for both cell shape and motility in normal as well as malignant cells (see e.g. Wessells *et al.* 1973, Willingham *et al.* 1977). Microtubules, which are involved in formation and maintenance of cell shape (see Porter *et al.* 1974) have a diameter of about 25 nm and are mainly composed of the protein subunit tubulin (Scheraga 1973). Microfilaments consist mainly of actin and constitute the contractile part of the cytoskeleton (see e.g. Wessells *et al.* 1973). They have a diameter of 5-6 nm. A characteristic feature is that they often assemble into bundles, which can be visualized by antibodies against actin, myosin, or tropomyosin (Gabbiani *et al.* 1973, Toh *et al.* 1976, Roszko *et al.* 1976). In addition,

filaments of about 10 nm in diameter are regularly observed in normal as well as neoplastic astrocytic cells and consist of the so-called glial fibrillary acidic protein (GFA) (Dahl and Bignami 1975).

In this investigation we have correlated ultrastructural characteristics with the presence and distribution of actin as demonstrated by indirect immunofluorescence. For comparison, the actin distribution of normal fetal brain cells at various stages of differentiation was also investigated.

MATERIALS AND METHODS

Animals and cell lines. Rats of the inbred BD IX-strain (Druckrey 1971) were used. Brains taken from fetuses on the 18th day of gestation were cut into small pieces and pressed through a steel mesh to obtain a cell suspension. Secondary fetal brain cells in culture were exposed for 48 hours to a partially purified protein fraction from adult pig brains, which caused flat epithelioid brain cells to differentiate into mature astrocytes. Homogenates of pig brains were ultracentrifuged, freeze dried, precipitated with alcohol and dialyzed as described by Lim & Mitsunobu (1975) (also called glia maturation factor).

Altogether 13 neoplastic cell lines were examined. Three cell lines (GVIC, NVIC, TVIC) originated from neuroectodermal tumours induced in the offspring by an intravenous pulse of ethylnitrosourea (ENU) (75 µg/g body weight) to pregnant BD IX rats on the 18th day of gestation. The cell lines were established from explanted tumours as described earlier (Larum & Rajewsky 1975).

The other type of permanent neoplastic cell lines (BTIC-BTIOC) originated from fetal brain cells transferred to monolayer cell culture shortly after exposure to ENU *in vivo* (Larum & Rajewsky 1975). GVIC, NVIC, TVIC and BTIC-BT7C were developed at the Max Planck Institut für Virusforschung, Tübingen, Germany (Larum & Rajewsky 1975) and BT8C-BTIOC were obtained in this laboratory. All cell lines were tumorigenic by s.c. reimplantation into BD IX rats, with the exception of BT8C where attempts to demonstrate tumorigenicity have so far been negative (>20 culture passages).

The cells were cultured in 60 mm plastic dishes (Cosar, California) in Eagle Dulbecco's medium (Flow, Glasgow) with a fourfold concentration of non-essential amino acids and 10% heat inactivated calf serum, penicillin (50 units/ml) and streptomycin (50 µg/ml), in a humidified atmosphere with 5% CO₂ in air at 37°C. All cell lines were regularly tested for mycoplasmas by aerobic and anaerobic incubation in agar medium, and only mycoplasma-free cells were used. When not otherwise specified, cells were fixed during late log-phase proliferation. For each cell line, specimens from 5-10 replicate experiments were investigated.

Transmission electron microscopy (TEM). Cultures were fixed for one hour in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 37°C, postfixed for 30 minutes in 1% OsO₄ in 0.1 M cacodylate buffer (pH 7.4) and dehydrated in increasing concentrations of

ethanol. *In situ* embedding in Epon 812 was performed by adding graded amounts of Epon-ethanol mixtures. The final polymerization was carried out at 40°C for 4 days. The Epon was then separated from the plastic dish by bending the dish. The cell monolayer remained in the Epon layer and could be viewed in the Epon sheet with a phase contrast microscope. Cells to be sectioned for electron microscopy were marked, and the scored area was cut out with a saw. This *in situ* embedding method allowed us to section the cells in a controlled manner, parallel or perpendicular to the substratum. Sections were cut on a Reichert ultratome Om U3. They were double-stained with uranyl acetate and lead citrate and examined in a Philips EM 300 electron microscope.

Immunofluorescence. Serum was taken from a patient with chronic active hepatitis, containing specific antibodies against smooth muscle (SMA-serum). The titre was 1/128. The serum was also tested for mitochondrial antibodies and showed negative reaction. SMA-serum from three other patients with chronic active hepatitis were also used in order to test the reproducibility of the results.

Cells on glass coverslips were washed twice with phosphate buffered saline (PBS), immersed in cold acetone at 4°C for 5 minutes, and air dried. The cells were then covered with SMA-serum diluted 1:8 to 1:10 in PBS and incubated at 17°C in a moist chamber for 30 minutes. Thereafter the preparations were washed three times in PBS and incubated for another 30 minutes in a 1:20 dilution of goat anti-human globulin fluorescein conjugate (Dako-immunoglobulin, Copenhagen, Denmark) washed three times in PBS and examined immediately by fluorescence microscopy.

Control preparations were treated before application of the conjugate with a) PBS or normal human serum b) SMA-serum neutralized by absorption with homogenates of smooth muscle from rat stomach, and c) hyperimmune serum (rabbit anti human IgG Behringwerke AG Germany). All these tests were negative and gave only low background fluorescence.

RESULTS

Transmission electron microscopy. These lines usually form several cell layers with a wicker-work pattern and many applied upw. foci. By light microscopy the cells were partly glass-like with many slender cytoplasmic processes, and partly flatter epithelioid, with short processes (see Larum *et al.* 1977). Giant cells were often seen. The histological appearance of the corresponding solid tumours, as well as other biological characteristics, is given in Table 1. The 3 V-lines and the 10 BT lines essentially showed the same characteristics by TEM. These included irregular shape of the nuclei with large indentations and condensed chromatin near the nuclear envelope and increased size of the nucleolus with characteristic microsegregations. The mitochondria contained fewer cristae than in

TABLE I Morphological Characteristics of Neoplastic Neurogenic Cell Lines

Cell line	Origin	Ploidy (semitine)	Filaments (10 nm)	Microtubules (25 nm)	Macrofilament bundles	Actin fluorescence	
						Bundles	Diffuse
V-lines	NVIC Peripheral neuroectoderm (pl leucomeningeal)	4.0	+	+	-	-	+
	GVIC Mixed glioma	2.0	+	+	-	-	+
	TVIC Tripartite neuroectoderm	2.0	+	++	+-	+	+
T-lines	BT1C G	2.0	+	+	+-	+	+
	BT2C G	4.2	+	++	+-	+	+
	BT3C G	6.4	+++	+	-	-	+
	BT4C N	3.0	+	+	-	-	+
	BT5C G	3.4	+++	+	-	-	+
	BT6C G	3.1	+	+	-	-	+
	BT7C N	2.6	+	++	+-	+	+
	BT8C -	2.0	+	++	+	+	-
	BT9Ca G	3.0	+	+	-	-	+
	BT10C G	4.0	+	++	+-	+	+

pretest, + moderate number, +++ high number, +- positive and negative cells

N, neuroectoderm-like tumours, G, anaplastic glioma like tumours (after retransplantation into syngeneic hosts).

normal cells and often had a swollen appearance, partly with bizarre structures. Glycogen granules were absent (Fig. 1). By cross-sectioning of the plasma membrane branched forms of microvilli as well as high pinocytotic activity could be seen (Figs 2, 3). Rounded cells had few short microtubules (Fig. 4), while flat epithelial cells contained many long microtubules (Fig. 5). In areas surrounding the controls numerous microtubules were observed (Fig. 6).

Generally microfilament bundles were found in cell lines which consisted mainly of flat, epithelial cells, but were reduced or absent in the lines consisting mainly of glia-like cells, i.e. round cell bodies and long cytoplasmic processes. In contrast to secondary fetal brain cells (see Halgren & Lærum 1978 b) there was a reduction of microfilaments in the periphery of the cells, except for BT8C and also TVIC where the areas of attachment to the substratum contained numerous microfilament bundles (Fig. 7). BT8C was not tumourigenic. Depending on the cell lines, varying numbers of 10 nm (glial) filaments were found. The highest numbers of glial filaments were found in strongly aneuploid cell lines, i.e. BT3C and BT5C (Table I, Figs 8-9). Otherwise there was no correlation

between histology, ploidy and the different components of the cytoskeletal system.

Demonstration of actin by immunofluorescence. Secondary fetal rat brain cells (18th day of gestation) were investigated as normal controls. These were epithelial and contained many longitudinal, parallel-oriented bundles of actin-positive filaments, conformal with the microfilament bundles as observed by TEM (see Gablanti *et al.* 1973; McNair *et al.* 1973; Trechev & Holborov 1976). After two days of exposure to glia maturation factor the fetal brain cells had undergone morphological differentiation into astrocyte-like cells, and the actin bundles had disappeared from the cell bodies. Instead, positive fluorescence in the peri-nuclear area was seen. Positive actin fluorescence was also found in the cytoplasmic processes (Figs. 10 a, b).

All malignant cell lines showed positive, though mainly diffuse, cytoplasmic fluorescence with lack of bundles. However TVIC exhibited strongly positive actin bundles, partly longitudinal and partly criss-cross. A similar pattern was present in the (non-tumourigenic) line BT8C. In the lines BT1C, BT2C, and BT7C some cells showed distinct bundles, but in most cells a general positive fluorescence over the whole cytoplasm was found.





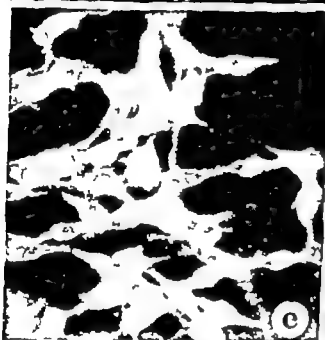


Fig 1 NVIC neuroblastoma cells in culture. The cytoplasm contains Golgi complexes, many free ribosomes, scattered cisternae of rough endoplasmic reticulum, 10 nm filaments and microtubules. $\times 18,000$. Inset shows glial filaments (10 nm) and microtubules in the cytoplasm of a NVIC cell. $\times 128,800$.

Fig 2 A NVIC neuroblastoma cell with branched microvilli on the surface. $\times 22,500$

Fig 3 A NVIC neuroblastoma cell containing small cytoplasmic vesicles, which are especially numerous along the cell margin. $\times 41,600$

Fig 4 A BT9C cell in culture. The cytoplasm displays only a few short microtubules (arrows). $\times 50,000$

Fig 5 Section through the region of a BT8C cell close to the substratum. Numerous long microtubules are seen. $\times 44,000$

Fig 6 Numerous microtubules surrounding the centriole in a BT9C cell. $\times 23,500$

Fig 7 Microfilament bundles in the cytoplasm of a BT8C cell. $\times 28,000$.

Fig 8 A BT3C cell containing numerous 10 nm (glial) filaments in the cytoplasm. $\times 56,700$

Fig 9 A GVIC-glioma cell in culture. The cytoplasm displays a few 10 nm filaments (arrow). $\times 66,000$

Fig 10 Actin in cultured cells demonstrated by immunofluorescence

- a) Secondary fetal brain cells. $\times 500$
- ii) Secondary fetal brain cells 48 hours after addition of brain extract. Note the disappearance of bundle structure. $\times 710$
- c) GVIC mixed glioma cells, and
- d) BT4C cells with strong perinuclear cytoplasmic fluorescence. $\times 500$
- e) BT8C cells with some actin bundles. $\times 570$
- f) TVIC neuroblastoma cells with actin bundles lying crosswise. $\times 500$.

Often the strongest fluorescence was found around the nucleus as a peri-nuclear halo. Positive fluorescence was commonly also seen over the nucleoli (Fig. 10 c-f).

Compared to secondary fetal brain cells, neoplastic cells possessed fewer actin bundles, which had a

more irregular pattern with bundles lying crosswise in all directions. Neoplastic cells with positive actin bundles were generally rather flat and epithelioid. Cells with diffuse cytoplasmic fluorescence were more rounded, or glia-like with slender processes, similar to what had been observed for microfilament bundles by TEM (see above).

However when compared to fetal brain cells with an astrocyte-like morphology (following exposure to the glia maturation factor) neoplastic cells with a glia like morphology were very similar i.e., with lack of bundles, except that they had a more strongly fluorescent peri-nuclear halo.

DISCUSSION

Ultrastructural characteristics of malignant cells have been extensively studied with transmission electron microscopy (for review see e.g., Hagmann 1969 and Borbeck 1976). This also applies to different types of malignant glioma cells either investigated as tumours *in vivo* (see Duffell *et al* 1963, Kumantshki *et al* 1973, Raimondi 1966, Laros 1972 and 1977) or in cell culture (Benda *et al* 1971, Sipe *et al* 1973 and 1974, Kroh *et al* 1973). The present study shows that ENU-induced neoplastic cells derived from the rat nervous system have essentially the same morphological appearance, regardless of whether malignant transformation takes place *in vivo* (as V-lines) or in cell culture (BT-lines). Observations include irregular nuclear shape, nuclear segregation, and giant nucleoli. Abnormal forms of mitochondria were also common, as earlier observed by other workers (Kroh *et al* 1973, Sipe *et al* 1973).

In addition, branched forms of microvilli were characteristic (see e.g. Gonda *et al* 1976). These coincided with changes in surface ultrastructure, which have been discussed in detail elsewhere (Haugen & Larum 1978a). Cell lines which contain numerous 10 nm filaments and fat vacuoles (BT3C ~ BT3C; Table 1) also have a high surface activity as evidenced by numerous microvilli, filopodia, ruffling membranes and zeiotic blebs (Haugen & Larum 1978a). From Table 1 it also appears that cell lines which gave rise to neuroblastoma-like tumours also contained varying amounts of 10 nm (glial) filaments.

However there is some discrepancy between the findings in the present neoplastic cell lines and those reported by other workers. Thus, Sipe (1973) did not find microfilaments in malignant gliomas. Although our neoplastic lines tended to show a reduced amount of peripheral microfilaments as compared to normal brain cells in culture, the filaments could still be observed in the cytoplasm.

Furthermore, Lantos (1977) in a study of solid tumours found that undifferentiated astrocytomas possessed many microtubules, but very few glial filaments. This author also observed an increased amount of glial filaments in highly differentiated gliomas and correspondingly few microtubules. It was thus claimed that in solid ENU-induced rat gliomas, the ratio of microtubules to glial filaments could be used as a criterion for the degree of differentiation. In our cell lines there was a certain tendency towards such a relationship although the correlation was rather weak (see Table 1). The high number of glial filaments seemed to be correlated with a correspondingly high degree of aneuploidy. Since the production of glial filaments *in vitro* may depend on culture conditions and tends to increase with the period of time the cells are kept in organ culture (Sipe *et al* 1973 and 1974) quantitative estimates of glial filaments are of limited value.

Normal glial cells generally contain fine parallel bundles of microfilaments at the base of the cells inside the plasma membrane (Brunk *et al* 1971; Wessels *et al* 1973). The organization of microfilament bundles as well as microtubules in cultures of fetal brain cells may also depend on the differentiated state of the cells. In secondary cultures these cells are mainly of a flat epithelioid (glial precursor) type, but can be induced to differentiate to an astrocyte-like phenotype by various means, such as cyclic AMP (Aloonen *et al* 1976) or a protein extracted from adult brain (Lim & Mitsunobu 1975). This leads to a rapid re-organization of the filament system in the whole cell. Microfilament bundles almost disappear from the plasma membrane near the substratum, and there is also a similar decrease in the perikarya (Lim *et al* 1977; Haugen & Larum 1978 b).

In neoplastic cells microfilament bundles are either reduced in number or totally absent (see Table 1). By immunofluorescence a corresponding reduction and partial loss of actin bundle structure was seen in the present malignant neurogenic cell lines. Instead a diffuse often strong fluorescence was found which tended to be strongest around the nucleus. When microfilament bundles were observed they were irregular, i.e. not oriented in parallel as in normal cells. The diffuse fluorescence is in accordance with observations in other cell systems undergoing malignant transformation (Pollack *et al* 1975; Wickus *et al* 1975). It has also been shown that the loss of actin bundles is correlated with acquisition of anchorage independent growth of virally transformed cells (Pollack & Rifkin 1975). The same correlation has been directly demonstrated by electron microscopy (McNutt *et al* 1973).

However, since practically the same pattern was

seen when fetal brain cells differentiated into astrocytes, this phenomenon appears to be more correlated with cell morphology and not with the neoplastic process itself. This is also in agreement with our findings in neoplastic cell lines, where the occurrence of microfilament bundles appeared to be correlated with cell shape and the relative area of anchorage to the substratum. Thus, rounded glial-like cells had a complete lack of actin bundles as well as microfilament bundles by TEM, while flat cells often contained both. This is also in accordance with a report by Williamson *et al* (1977), who concluded that the distribution of microfilament bundles is related to both adhesiveness to the substratum and to cell shape. Recently Tok *et al* (1977) found that astrocytomas showed enhanced expression of actin as compared to normal astrocytes. This was partly confirmed by our observations, where malignant cells had a strong perinuclear fluorescence.

In our experiments cells which were round in shape demonstrated fewer and shorter microtubules than the cells which were flatter and polymorphic. This is in accordance with the report by Brubaker *et al* (1975), and Edelman & Yahara (1976). The transformed cells contained very few short and crossing cytoplasmic microtubules. Miller *et al* (1977) also reported that the area surrounding the centrosome contained very few microtubules, but this could not be verified in the present study (see Fig. 6).

Apart from this, there seemed to be no clear correlation between internal organization of the cell and different biological parameters such as ploidy, histology and general growth pattern. Different morphological variants of normal as well as neoplastic cells appear to be characterized by corresponding changes of the cytoskeleton complex, pointing to their importance for the regulation of cell shape. However, when directly comparable cell types are investigated, the differences between normal and neoplastic cells in culture seem to be more quantitative than qualitative.

This investigation was supported by the Norwegian Cancer Society. We thank Mrs Eva Bohlen and Miss Gre Oldervang for expert technical assistance. We are also greatly indebted to Dr Orlin Bee for mycoplasma tests. We thank Professor H. Dahlen at the laboratory for electron microscopy for valuable advice and discussions.

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HISTOCHEMICAL ENZYME ACTIVITY CORRELATED TO THE STRUCTURAL SEGMENTATION OF THE PROXIMAL CONVOLUTED TUBULE IN SALT DEPLETED AND SALT LOADED RAT KIDNEYS

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Nørgaard, Tove. Histochemical enzyme activity correlated to the structural segmentation of the proximal convoluted tubule in salt-depleted and salt-loaded rat kidneys. *Acta path. microbiol. scand. Sect. A*, 86: 427-435 1978.

In salt-depleted and salt-loaded rat kidneys a study was made of the structural segmentation of the proximal convoluted tubule (PCT) and the histochemical activity of non-specific acid and alkaline phosphatases and succinate dehydrogenase in the same segments. No quantitative structural or segmental alterations were observed, but significant changes in enzyme activity occurred. These comprised: 1) A decrease in activity of acid phosphatase in segment 1 and the transitional zone in salt-depleted kidneys, and an increase in enzyme activity in segment 2 in salt-loaded kidneys, 2) a decrease in alkaline phosphatase activity in segment 2 in both salt-depleted and salt-loaded kidneys and 3) a decrease in succinate dehydrogenase activity in segment 2 in salt-depleted kidneys, and an increase in activity in the same segment in salt-loaded kidneys. Thus long-term variations in sodium intake are followed by segment-correlated variations in the activity of acid and alkaline phosphatase and succinate dehydrogenase in the PCT.

Key words: Kidney tubule, proximal, histochemistry, kidney enzymes, salt-depletion, salt-load.

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Received 15/78 Accepted 28/7/78

In a previous histochemical study on the normal rat kidney relating to enzyme activity in the different segments of the proximal convoluted tubule (PCT), segment-correlated variations in the activity of non-specific acid and alkaline phosphatases and succinate dehydrogenase were found (Nørgaard 1976). Acid phosphatase is known to be associated with lysosomal activity and protein reabsorption, succinate dehydrogenase with the oxidative metabolism whereas the functional significance of alkaline phosphatase is questionable. It is, however, generally accepted that it takes part in reabsorption processes.

Alterations in sodium balance cause changes in renin-angiotensin activity. Injections of these hormones cause variations in e.g. renal protein and

glucose excretion (Rosenfeld *et al.* 1965, Barrackough *et al.* 1967). These changes in renal function might involve functional changes in the proximal tubules. Therefore the aim of the present study was to see whether salt-depletion or salt-load would induce changes in the segmentation of the PCT or in the histochemically demonstrable activity of these three enzymes in the different segments.

MATERIALS AND METHODS

Salt-depleted rats Five ten-week-old white male rats each weighing approximately 200 gm were given a sodium deficient diet (ICN Pharmaceuticals, 902902) and distilled water *ad libitum* for four weeks.

Salt-loaded rats Over the same period five male rats of the same strain, age and weight were given ordinary

rat pellets (KFK 7312) and distilled water to which 1 gm. sodium per 100 ml. was added.

Control rats Five control rats were given ordinary rat pellets and tap water for the same length of time. All rats were placed on wire netting to prevent contamination with sodium from urine, faeces and litter. After four weeks all the rats were anaesthetized by intraperitoneal injection of sodium pentobarbital 100 mg. per kg. body weight, and a left-sided nephrectomy was performed. The kidneys were instantly frozen in isopentane cooled to -160°C as previously described (Norgaard 1976). 4 μm serial sections were cut from three zones of the renal cortex (superficial - intermediate - and juxtamedullary) on a cryostat at -20°C . The sections were taken alternately for morphological and enzyme histochemical studies. The enzymes investigated were non-specific acid and alkaline phosphatases and succinate dehydrogenase. The morphological and histochemical methods used and the morphological basis for dividing the PCT into segment 1 the transitional zone and segment 2 were the same as those previously described (Norgaard 1976). The scale for semiquantitative grading of enzyme activity into slight or heavy in both experimental and control kidneys

was identical with the scale previously used (Norgaard 1976). As a check on enzyme reactions in the control group all results were compared with new sections from the normal kidneys previously described. As the control kidneys in this study appeared identical with the previously described normal kidneys, these were used as normal material in this study. From each kidney 3-5 pairs of sections were investigated to determine the enzyme activity in the kidney. The results from the different sections were in accordance with one another. The distribution of observations between the different kidneys is seen in Table 1.

As a control for succinate dehydrogenase activity coenzyme Q_{10} was added to the incubation medium (Andersen & Haver 1973) and serial sections were alternately incubated in media with and without coenzyme Q_{10} . The enzyme activities in adjacent sections were then compared. Additional frozen sections from all kidneys were stained with Hematoxylin-Eosin as a light microscopy control.

The results were analysed statistically using the low fold table test.

TABLE 1 Mean Values of High Enzyme Activity in Different Segments of the PCT. Abbreviations as in Fig. 2

	zone	segment	Acid Phosphatase			Alkaline Phosphatase			Succinate Dehydrogenase	
			mean	SEM	N	mean	SEM	N	mean	SEM
Salt depleted rats	Superficial	A	0	0	5	88	8.8	2	100	4
		B	8	3.7	5	70	7.2	5	90	4.8
		C	22	7.3	5	67	3.0	5	62	2.4
		D	41	4.1	5	46	4.6	5	30	4.5
	Intermediate	A	0		5	93	3.8	5	100	4
		B	3	2.2	5	85	1.2	5	95	2.0
		C	9	3.5	5	63	7.6	5	77	4.5
		D	43	2.7	5	54	4.3	5	25	4.8
	Juxta-medullary	A	0		5	100		3	100	3
		B	1	1.0	5	99	6.7	4	100	3
		C	7	4.4	5	87	8.2	4	83	9.7
		D	17	9.5	5	68	4.3	5	31	10.1
	Superficial	A	0		4	100		3	100	4
		B	4	1.6	5	84	7.7	5	88	4.2
		C	19	6.8	5	71	9.5	5	66	10.7
		D	26	2.6	5	52	6.6	5	54	7.0
Salt loaded rats	Intermediate	A	0		3	88	10.6	4	100	4
		B	11	3.2	5	91	4.4	5	96	4.2
		C	12	3.7	5	80	6.5	5	77	12.6
		D	25	3.5	5	57	5.5	5	50	7.7
	Juxta-medullary	A	0		3	100		3	100	4
		B	5	4.0	3	89	3.5	4	97	2.4
		C	11	4.8	3	82	7.1	4	83	9.7
		D	20	2.8	3	84	4.0	4	63	6.8

N: Number of rats investigated.

RESULTS

Structure of the PCT In freeze-dried sections, the structure of the cells in the PCT in salt-depleted and

salt-loaded kidneys was identical with that in normal kidneys. The tubular cells in segment 1 had a granular appearance, those in segment 2 showed radial striation and in the transitional zone cells

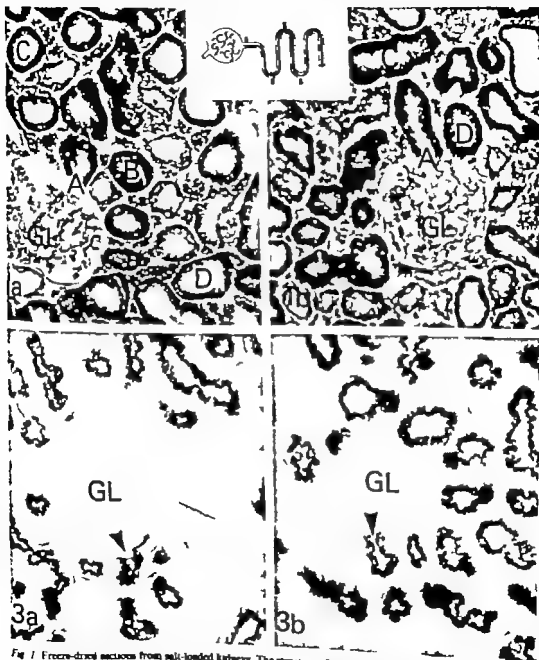


Fig 1 Freeze-dried sections from salt-loaded kidneys. The structure of the PCT seen most often in the kidneys under test was similar to the normal structure (1a). The only exception was found in one nephron in a salt-loaded kidney where the PCT at the urinary pole appeared similar to that of the transitional zone, in which both granulation and striation in the cytoplasm were seen (1b). Urinary pole (A), segment 1 (B), transitional zone (C) and segment 2 (D). Inset: schematic drawing of the segmentations of the PCT ($\times 200$).

Fig 3 Non-specific alkaline phosphatase activity in salt-depleted kidneys. The enzyme activity at the urinary pole was mostly heavy (3a), but a few nephrons showed slight activity at the urinary pole (3b) ($\times 200$).

TABLE 2. Distribution of Segment 1 the Transitional Zone and Segment 2 of the Proximal Cortical Tubule in Salt-Depleted Salt loaded and Normal Rats, Expressed as a Percentage of Cross Sectioned Tubules

	Rat No	Superficial zone		translational zone		Intermediate zone		Juxtamedullary zone	
		seg-ment 1	seg-ment 2	seg-ment 1	seg-ment 2	seg-ment 1	seg-ment 2	seg-ment 1	seg-ment 2
Salt depleted rats	13	30	10	60	27	10	63	26	11
	15	24	10	66	26	7	67	26	13
	16	23	10	67	27	10	63	30	9
	17	24	11	65	24	5	71	22	9
	18	25	12	62	24	8	68	25	9
	Mean value %	25	10	65	26	8	66	26	10
Salt loaded rats	22	26	13	61	28	11	61	28	8
	23	26	13	61	27	13	60	26	8
	24	29	12	59	31	12	57	31	7
	41	30	11	59	27	11	62	27	12
	42	33	11	59	31	9	60	34	12
	Mean value %	29	12	59	29	11	60	29	9
Normal rats	Mean value %	28	11	61	28	12	61	23	9

The values are very similar in the kidneys under test and the normal kidneys. In addition similar values are seen in all cortical zones. The values for the normal rat kidneys are taken from a previous study (Aagaard 1976)

with both granular and striated appearance were seen (Fig. 1a). The tubular cells at the urinary pole were consistently granular in appearance except in one nephron in a salt loaded rat (no. 22) where the cells resembled those in the transitional zone (Fig. 1b). In the kidneys from salt-depleted and salt loaded rats the distribution of segment 1 the transitional zone and segment 2 expressed as a percentage of the total number of cross sections of the PCT was similar to that found in the normal kidneys (Table 2).

Acid phosphatase The activity of non-specific acid phosphatase in the PCT of the salt-depleted and salt loaded kidneys appeared of the same type as in the normal kidneys, i.e. a granular or spotty precipitate localized at the basal part of the cells. Enzyme activity could be graded into slight or heavy. The activity pattern in the salt-depleted and salt-loaded PCT was principally similar to that in the normal kidney. The activity at the urinary pole was thus consistently slight and an increasing percentage tubular cells had heavy enzyme activity from segment 1 to segment 2 (Fig. 2). However

some significant differences in enzyme activity in the PCT of salt-depleted, salt loaded and normal kidneys were seen. In salt-depleted kidneys compared with normal kidneys there was a decrease in enzyme activity in segment 1 in the superficial and intermediate cortical zones, and in the transitional zone of the PCT in the intermediate and juxtamedullary cortex (Fig. 6). In salt loaded, compared with normal kidneys, there was a decrease in enzyme activity in segment 1 in the superficial cortex, the transitional zone in the intermediate and juxtamedullary zones of the cortex, and segment 2 in all cortical zones (Fig. 6). The only significant difference in enzyme activity between salt-depleted and salt-loaded kidneys was a higher enzyme activity in segment 2 in all cortical zones in the salt-depleted kidneys (Fig. 6).

Alkaline phosphatase As in normal kidneys this enzyme was localized in the brush border of the PCT in the salt-depleted and salt loaded kidneys, and the activity could be graded into slight or heavy. In salt-depleted kidneys a few nephrons in the superficial and intermediate zones, and in salt

ACID PHOSPHATASES

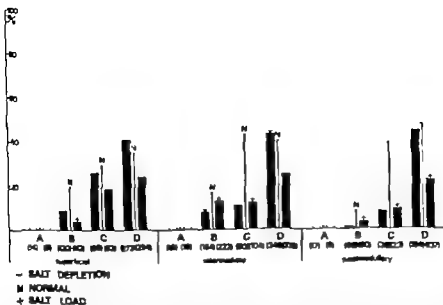


Fig 2 Diagram showing the percentage of different segments with heavy acid phosphatase activity in superficial, intermediate and juxtamedullary nephrons in salt-depleted, normal and salt-loaded kidneys. An increase in tubular cells with heavy enzyme activity is seen throughout the course of the PCT in both salt-depleted, normal and salt-loaded kidneys. The enzyme patterns are tubular in all cortical zones. Urinary pole (A), more distal in segment 1 (B), transitional zone (C), segment 2 (D). Figures in brackets are the number of tubules investigated.

ALKALINE PHOSPHATASES

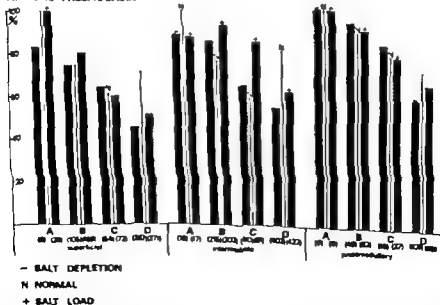


Fig 4 Diagram showing the percentage of different segments with heavy alkaline phosphatase activity in superficial, intermediate and juxtamedullary nephrons in salt-depleted, normal and salt-loaded kidneys. A decrease in tubular cells with heavy enzyme activity is seen throughout the course of the PCT in the three different groups of kidneys. The enzyme patterns are similar in all cortical zones. Abbreviations are the same as in Fig. 2.

loaded kidneys a few nephrons in the intermediate zone showed slight enzyme activity at the urinary pole (Fig. 3). This was not seen in the normal PCT. Morphologically these nephrons appeared to have segment 1 type cells at the urinary pole. The majority of nephrons however did display a heavy enzyme activity at the urinary pole (Fig. 4). The enzyme activity pattern in the PCT of salt-depleted and salt-loaded kidneys was otherwise similar to the normal pattern with a decreasing percentage of tubular cells showing heavy enzyme activity from segment 1 to segment 2 (Fig. 4). The difference in enzyme activity between segment 1 and segment 2 in each of the two groups under test was statistically significant in contrast to the difference in activity in these two segments in the normal kidneys. The only significant difference in enzyme activity between salt-depleted and normal kidneys was a decrease in segment 2 in the superficial and intermediate cortex of the salt-depleted kidneys. Enzyme activity in the PCT of salt-loaded and normal kidneys showed a significant decrease in enzyme activity in segment 2 in the superficial and intermediate cortex when compared with normal kidneys. The only significant difference in enzyme activity between salt depleted and salt-loaded kidneys was a lower

enzyme activity in the transitional zone of the PCT in the intermediate cortex in salt-depleted kidneys (Fig. 6).

Succinate dehydrogenase As in normal kidneys the activity of this enzyme was scattered diffusely in the cytoplasm of cells in the PCT of both salt-depleted and salt loaded kidneys. Activity could be graded into slight or heavy. Addition of coenzyme Q₁₀ to the incubation medium made no difference in enzyme localization but there was an increase in reaction velocity. Any differences in the grade of enzyme activity in corresponding sections were strictly maintained whether coenzyme Q₁₀ was added or not. In salt-depleted and salt-loaded kidneys the enzyme pattern along the PCT was similar to normal, the enzyme activity at the urinary pole was consistently heavy and there was a significant decrease in tubular cells showing heavy enzyme activity from segment 1 to segment 2 (Fig. 5). Comparing salt-depleted and normal PCT there was a significant increase in enzyme activity in the transitional zone of nephrons in the intermediate cortex and a significant decrease was seen in segment 2 in the superficial and juxtamedullary cortex of the same salt-depleted group. There were significant differences between salt loaded and

SUCCINATE DEHYDROGENASE

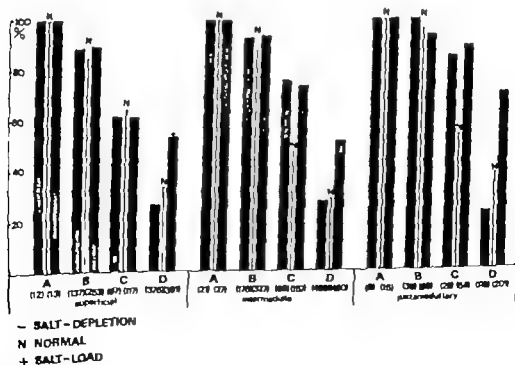


Fig. 5 Diagram showing the percentage of different segments with heavy succinate dehydrogenase activity in salt-depleted, normal and salt loaded kidneys. A decrease in tubular cells with heavy enzyme activity is seen throughout the course of the PCT in all three groups of kidneys. The enzyme patterns are similar in all cortical zones. Abbreviations are the same as in Fig. 2.

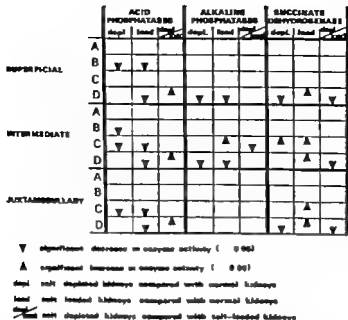


Fig 6 Comparison of the statistically significant variations ($p < 0.05$) in activity of non-specific acid and alkaline phosphatases and succinate dehydrogenase in the different segments of the PCT as the superficial intermediate and juxtamedullary cortex of salt-depleted normal and salt-loaded kidneys

Acid phosphatase A decrease in the activity of acid phosphatase is constantly found in segment 1 and/or the transitional zone in salt-depleted kidneys, whereas a constant decrease is found in segment 2 in salt-loaded kidneys. Comparing salt-depleted and salt-loaded kidneys a constantly higher enzyme activity was found in segment 2 in the former.

Alkaline Phosphatase During both salt-depletion and salt-load a decrease in enzyme activity was found in segment 2. The only significant difference between salt-depleted and salt-loaded kidneys was found in segment 2 in the intermediate cortex, where a lower enzyme activity was seen in the salt-depleted kidneys.

Succinate dehydrogenase During salt-depletion a decrease in enzyme activity was found in segment 2 in the superficial and juxtamedullary zones, during salt-load an increase was found in the transitional zone and segment 2. Comparing salt-depleted and salt-loaded kidneys the former consistently showed a lower enzyme activity in segment 2.

normal kidneys. An increase in enzyme activity was found in the transitional zone in the intermediate and juxtamedullary cortex and in segment 2 in all cortical zones. Comparing salt-depleted and salt-loaded kidneys a significantly lower enzyme activity was seen in segment 2 in all cortical zones in the salt-depleted kidneys (Fig 6).

DISCUSSION

The aim of the present study was to show whether dietary salt-depletion or salt-load would result in 1) a quantitative alteration in the segmentation of the PCT in the rat kidney as evaluated in freeze-dried sections, or 2) changes in the histochemically demonstrable activity of non-specific acid and alkaline phosphatases and succinate dehydrogenase in the different segments of the PCT.

Segmentation of the PCT The freeze-dried

structure of the PCT was essentially unchanged during salt-depletion and salt-load. In one nephron, however the structure of the PCT at the urinary pole was similar to the structure of the transitional zone. The relative lengths of the different segments, as evaluated by the relative number of cross sectioned segments, were also the same, independent of cortical level. Thus no significant morphological alterations were induced by either salt depletion or salt-load.

Enzyme activity in the different segments of the PCT The activity of all three enzymes investigated changed significantly during both salt-depletion and salt-load. At the same time a close correlation between the changes in enzyme activity and the structural segmentation of the PCT could be demonstrated. The changes in enzyme activity were similar in all cortical zones.

The activity of acid phosphatase which correlates

to lysosomal activity and protein reabsorption (Siraus 1962) (Maunsbach 1966), decreased in both salt-depleted and salt-loaded rats. These changes in enzyme activity were seen, however in different parts of the PCT in the two states (Fig. 3) This would seem to indicate that salt-depletion as well as salt load may induce a decrease in lysosomal activity and protein reabsorption, and that the segment of the PCT involved depends on whether sodium balance is positive or negative. It has been shown that acute sodium depletion in rats is associated with increased proteinuria (Tobian & Nason 1966) The physiological explanation for this is unknown, but the results in this study suggest that a decrease in tubular reabsorption may contribute to the proteinuria. Although no other histochemical study on the activity of acid phosphatase in the PCT during salt-load or salt-depletion has been published Cremers van Dijk (1973) found a decrease in activity of acid phosphatase in the outer cortex (presumably corresponding to the superficial zone in this study) in potassium deprived sodium loaded rats. These results were obtained in a biochemical study on kidney homogenates As a main part of the outer cortex is composed of proximal convoluted tubules the decrease in enzyme activity found in the biochemical study seem to be in accordance with the results in the present study

Alkaline phosphatase is generally accepted as participating in cellular reabsorption processes. Its participation in glucose reabsorption has been discussed (Schmidt & Dubach 1969) In this study a decrease in alkaline phosphatase activity was seen in all cortical zones during salt-depletion and salt load. This decrease was only significant in the superficial and intermediate zones (Fig. 3) The changes in enzyme activity were localized to segment 2 in both states. This decrease in enzyme activity may indicate that reabsorption in this part of the nephron decreases during salt load and salt-depletion. It is well known that salt-depletion leads to a rise in renin and angiotensin activity. Injections of these hormones cause glucosuria (Barracrough et al 1967) and angiotensin has been suspected of causing a decrease in tubular reabsorption of glucose (Hughes Jones et al 1949) The decrease in alkaline phosphatase during salt-depletion may thus be correlated to the decrease in tubular reabsorption. No other histochemical study on the activity of alkaline phosphatases during salt-load and salt-depletion has been reported, but in a biochemical study on kidney homogenates from potassium deprived sodium-loaded rats, a decrease in enzyme activity was found. This was the same in the superficial intermediate and deep parts of the renal cortex (Cremers van Dijk 1973).

Succinate dehydrogenase is involved in oxidative metabolism and the activity of this enzyme can be considered as an indicator of oxidative metabolic rate. Addition of coenzyme Q_{10} to the incubation medium accelerated the reaction velocity but the variations in succinate dehydrogenase activity observed in different segments were maintained. Therefore, the differences in the histochemically demonstrable activity of succinate dehydrogenase in the PCT are not due to lack of coenzyme Q_{10} .

During salt-load and salt-depletion the activity of succinate dehydrogenase was found to increase and decrease respectively in segment 2 of the PCT. The increase in enzyme activity during salt-load was found in all cortical zones. One may expect that changes in the activity of succinate dehydrogenase may be followed by parallel changes in oxidative metabolism. Oxidative metabolism in the PCT is associated with the reabsorption of sodium (Weinstein & Klose 1969) (Weinstein 1970) which is responsible for fluid reabsorption in the PCT. Micropuncture studies have shown that fluid reabsorption rate in the PCT is unchanged during salt load (Horster & Thurnau 1968), (Hanslet 1967). This indicates that sodium reabsorption in the PCT does not change during longterm variations in sodium balance. The changes in succinate dehydrogenase activity thus seem unrelated to sodium reabsorption and an explanation of the metabolic changes is not possible at present.

In conclusion Longterm salt-depletion and salt load leads to: 1) no morphological change in the relative length of the different segments of the PCT and 2) segment-correlated changes in the activity of non specific acid and alkaline phosphatases and succinate dehydrogenase. The most constant changes in enzyme activity comparing salt-depleted and salt loaded kidneys, were seen in segment 2 with regard to the activity of acid phosphatases and succinate dehydrogenase, in which the changes in enzyme activity were similar in all cortical zones. The physiological significance of these changes in enzyme activity is not clear at present.

This work was supported by grant from *Hjerteforeningen* Copenhagen

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In conclusion. Longterm salt-depletion and salt load leads to 1) no morphological change in the relative length of the different segments of the PCT and 2) segment-correlated changes in the activity of non-specific acid and alkaline phosphatases and succinate dehydrogenase. The most constant changes in enzyme activity comparing salt-depleted and salt-loaded kidneys, were seen in segment 2 with regard to the activity of acid phosphatases and succinate dehydrogenase, in which the changes in enzyme activity were similar in all cortical zones. The physiological significance of these changes in enzyme activity is not clear at present.

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A RETROSPECTIVE HISTOLOGICAL STUDY OF 669 CASES OF PRIMARY CUTANEOUS MALIGNANT MELANOMA IN CLINICAL STAGE I

1. Histological Classification, Sex and Age of the Patients, Localization of Tumour and Prognosis

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Larsen, T. E. & Grude, T. H. A retrospective study of 669 cases of primary cutaneous malignant melanoma in clinical stage I. 1. Histological classification, sex and age of the patients, localization of tumour and prognosis. *Acta path. microbiol. scand. Sect. A*, 86: 437-450, 1978.

A selected series of 669 primary malignant melanomas of the skin, stage I, has been classified according to Clark's system into lentigo maligna melanoma (86), superficial spreading malignant melanoma (259), nodular malignant melanoma (194) and undifferentiable malignant melanoma (130). It was often difficult to distinguish between lentigo maligna melanoma and superficial spreading malignant melanoma, and sometimes also between this last type and nodular melanoma. There seem to be borderline cases between the respective types. The 10-year specific curative survival rate (~ the cure rate) was 98.3% for the lentigo maligna melanoma, 78.6% for nodular malignant melanoma and 74.7% for the undifferentiable group of melanomas. The 5-year observed prognosis of the 3 main types is satisfactory compared with other investigations. As the prognosis of the 3 types of cutaneous malignant melanoma differs considerably the use of this classification is recommended. The number of undifferentiable cases is likely to be reduced in the routine work when several sections of each tumour are studied.

Key words: Melanoma, classification, prognosis.

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Received 20. 7. 78. Accepted 18. iv. 78.

A rapid rise in the incidence of malignant melanoma of the skin has been reported from many parts of the world (Etnood & Lee 1975). This rise has also been seen in Norway (Magnus 1975), where the annual rate of new cases rose from about 2 per 100 000 population in 1955 to almost 8 in 1973 (Fig. 1).

Magnus also found a «cohort» effect with the highest increase among Norwegians born 1900-1935. The change was most striking for melanomas

occurring on the male trunk and the female leg while the incidence on the face and the foot is almost unaltered. He suggested that this is the result of changed clothing and recreational habits during the period from the first world war to after the second world war. This is in agreement with the results of investigations on the Canadian and Swedish populations (Etnood *et al.* 1974, Ericson *et al.* 1976) and partly also with that on the Finnish population (Pukkunen 1977a).

During recent years there has been new interest

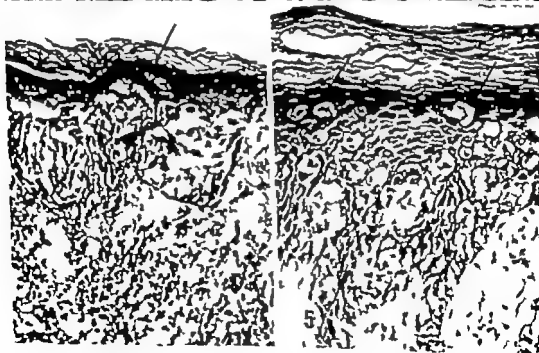
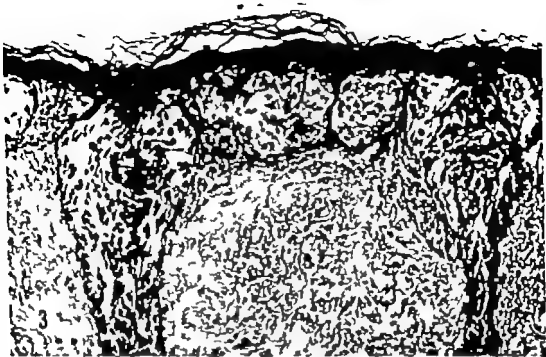


Fig 3 Lentigo maligna. Early invasive growth may be present. H&E with saffron. Orig. magn. $\times 40$.

Fig 4 Lentigo maligna melanoma, early stage. Arrow indicates a single tumour cell in the middle of an atrophic epidermis. H&E with saffron. Orig. magn. $\times 40$.

Fig 5 Preinvasive superficial spreading malignant melanoma. Pagetoid growth of tumour cells in a thickened epidermis (arrows). H&E with saffron. Orig. magn. $\times 40$.

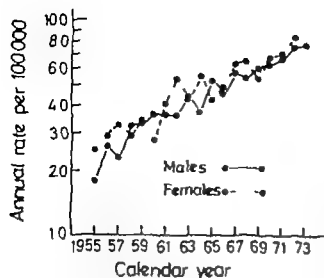


Fig 1 The age-adjusted incidence rates of cutaneous malignant melanoma in Norway 1955-1973 (from Magnus 1975)

in the histological classification of melanomas with subdivision into three different types, each with a different prognosis. Typing has therefore become important both because it has a bearing on prognosis and also because it influences the choice of treatment.

A retrospective study was started in 1974 with the object of examining about 1 000 specimens of melanoma from the different pathological laboratories in Norway.

We asked ourselves the following questions.

A. Is it possible to apply the criteria for the

histological classification of melanomas according to Clark (1967) to routine histological sections collected from a number of pathological laboratories?

B. Which are the main differential diagnostic problems?

C. What is the incidence of each of the different types in our series and is it likely to reflect that of the total population of Norwegian melanomas?

D. What is the prognosis of the different types in our series and how does it compare with investigations from other countries?

Clark was the first to point out that in addition to what he called *lentigo maligna melanoma*, i.e. a malignant melanoma developed in a lentigo maligna, (Fig 2-4) there seemed to be two other clinically and histologically different types of melanoma. He rejected that the junctional nevi should play any part in the development of these melanomas and introduced the terms *superficial spreading melanoma* (Fig 5-6) and *nodular melanoma* (Fig 7) (Clark 1967 Clark et al. 1969).

McGovern (1970) supported Clark's histological classification but could not agree with his theory of histogenesis and preferred a somewhat different terminology.

At the combined National Cancer Conference/VIIth International Pigment Cell Conference in Sydney 1972 the problem of terminology was provisionally solved by a compromise (McGovern et al. 1973).

Some different variations in terminology are shown in Table 1.

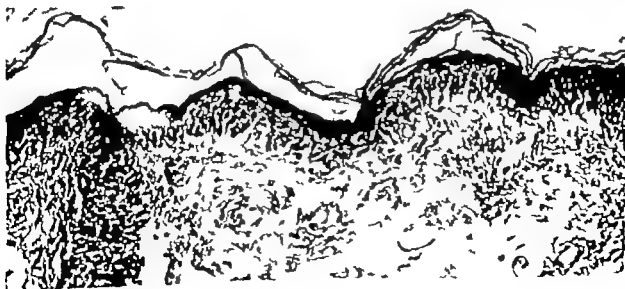


Fig 2 Early lentigo maligna. H&E with saffron. Orig. magn $\times 40$

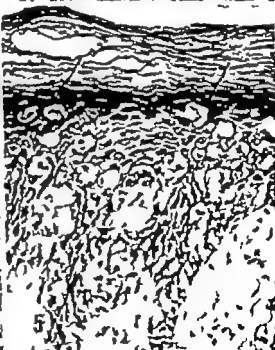
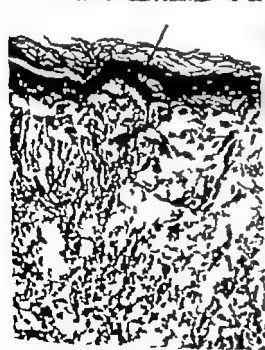
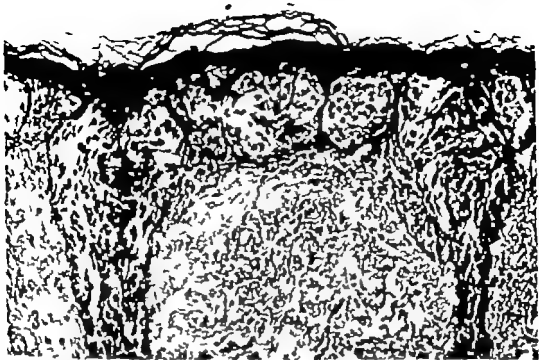


Fig 3 Low leucopo melanoma. Early invasive growth may be present. H&E with saffron. Orig. magn. $\times 40$.

Fig 4 Leucopo melanoma nodulosa, early stage. Arrow indicates a single tumour cell in the middle of an atrophic epidermis. H&E with saffron. Orig. magn. $\times 40$.

Fig 5 Proliferative superficial spreading malignant melanoma. Pagetoid growth of tumour cells in a thickened epidermis (arrows). H&E with saffron. Orig. magn. $\times 40$.



Fig 6 Superficial spreading malignant melanoma. Seven rete ridges are affected preinvasively (to the right). Arrows indicate Pagetoid growth of single tumour cells intraepidermally. H&E with saffron. Orig. magn. $\times 15$



Fig 7 Nodular malignant melanoma. No preinvasive growth phase. H&E with saffron. Orig. magn. $\times 40$.

TABLE 1 *Terminology of Malignant Melanomas*

Clark et al (1969)	Lentigo maligna melanoma	Superficial spreading melanoma	Nodular melanoma
McGowen (1970)	Melanoma arising in Hutchinson's melanotic freckle	Melanoma arising as premalignant melanosis	Nodular melanoma
Sydney conference 1972 (McGowen et al 1973)	Melanoma, invasive, with adjacent intraepidermal component of Hutchinson's freckle type	Melanoma, invasive with adjacent intraepidermal component of superficial spreading type	Melanoma, invasive, without adjacent intraepidermal component
WHO 1974 (Seidman et al 1974)		Malignant melanoma arising in precancerous melanosis, including Hutchinson's melanotic freckle	Malignant melanoma

In this paper the following terms will be used.
 Benign naevus (BN)
 Malignant melanoma of the skin (MM)
 Primary malignant melanoma of the skin (PMM)
 Lentigo maligna (LM)
 Lentigo maligna melanoma (LMM)
 Premalignant superficial spreading malignant melanoma (Premalignant SSMM)
 Superficial spreading malignant melanoma (SSMM)
 Nodular malignant melanoma (NMM)
 Unclassifiable malignant melanoma (UMM)
 Metastases from malignant melanoma (Met)

OWN SERIES

Selection

Since 1952 the compulsory reporting of all malignant tumours to the *Cancer Registry of Norway* makes it possible to get a reliable picture of the incidence of MM in the Norwegian population. We have made a retrospective investigation of a part of the series used by Metcalf (1973) which was based on all 2341 cases registered as PMM by the Cancer Registry from 1955-1972.

Approximately 1000 cases registered as PMM and 300 cases registered as LM totally 1321 cases, were extracted randomly by the Cancer Registry. The specimens had originally been examined by pathologists in 8 laboratories all over the country, but 3 of the laboratories covered, however more than 75% of all cases.

Fig. 8 shows the traditional way of taking sections from skin tumours used by Norwegian pathologists. The number of sections depends on the size of the tumour.

We prepared 1 unstained slide from each of what was supposed to be the most representative paraffin blocks. These sections were sent to The Institute of Pathology Rikshospitalet and stained with haematoxylin/eosin/saffron. They were given a random code number and circulated among the authors and Professor O H

Jervest, and studied without access to any clinical information.

At first we had to decide whether the section represented a PMM. If so, further classification into LM, SS, SVM, NMM, or UMM was made. Our individual judgements were compared at weekly meetings. In case of disagreement the tumour was reviewed by all of us and discussed. If 2 or 3 of us agreed the case was considered classifiable. If we all disagreed, it was registered as unclassifiable.

Table 2 clearly illustrates some of the disadvantages of a retrospective investigation. In 56 specimens no tumour tissue was left in the paraffin block, while 127 consisted of skin and lymph node metastases. 199 cases were sent possible to judge either because the lack of a clear epidermal origin of the tumour made it difficult to decide whether it was primary or metastatic or because of poor technical quality of the section. In 55 cases we found a benign melanotic tumour. In 7 cases we found a pyramide

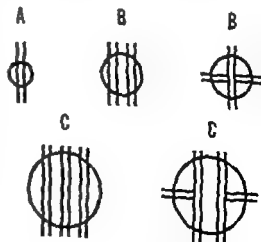


Fig. 8 A schematic illustration of macro-sectioning of a malignant melanoma of small (A), medium (B) and large (C) size.

TABLE 2 *Original Series*

Histological diagnosis	No. of cases
Primary malignant melanoma	825
Metastasis from malignant melanoma	127
Lentigo maligna	39
Benign naevus	55
Juvenile melanoma (Spitz naevus)	7
Other malignant tumour	9
Other benign tumour	4
No tumour	56
Not possible to judge	199
Total number of cases studied	1321

melanoma (Spitz naevus) 13 examples of benign or malignant non melanous tumour were found.

This left us with 39 cases of LM and 825 cases of PMM. Among these 825 cases, 669 were according to the Cancer Registry records, in clinical stage I i.e. no sign of metastases had been found at the time of registration. Among this latter group each paraffin block contained a various number of tumour sections: 350 cases were represented by 1 section, 172 cases by 2 sections, 137 cases by 3 sections and 10 cases by 4 sections. These 669 cases in clinical stage I were used in study prognosis and only these will be referred to below.

Classification

Table 3 shows the distribution of the different types e.g. 86 LMM, 259 SMM, 194 NMM and 130 UMM. Since our cases are selected this distribution does not necessarily reflect the total population of Norwegian MM.

Sex and Age

The sex and age distribution is seen in Table 4. There are 256 (38.3%) men and 413 (61.7%)

women. The dominating age groups for both sexes are 30-70 with a maximum at 40-50 years. This is approximately the same as was found by *Magnus* and in several other investigations (*Malcu* 1970, *MacDonald et al.* 1971, *Hauss & Proppe* 1972, *Davis* 1976, *Ericsson et al.* 1976 and *Pakkanen* 1977a).

Table 4 shows further sex and age distribution for each of the different tumour types. Most patients of both sexes with LMM are older than 50 years. There are, however, 12 patients between 20 and 40 years of age with LMM. Most men with NMM are also older than 50 years. Most patients of both sexes with SMM are under 50 years of age. Further 18 patients are 6-19 years old (12 NMM, 4 UMM and 2 SMM).

Localization

The regional distribution is shown in Table 5. It is seen that in the male the MM on the trunk and in the female those on the leg were the most frequent, which is similar to what was described by *Magnus* and in several other publications (*Malcu* 1970, *Hauss & Proppe* 1972, *Franklin et al.* 1975, *Davis et al.* 1976, *Ericsson et al.* 1976 and *Pakkanen* 1977a).

The regional distribution of the different types of MM is shown in Table 6. It illustrates that in patients with LMM the head is the most frequent site. Most of the SMM are found on the trunk or on the leg. The trunk is also the most frequent site for NMM followed by the head. Our unclassifiable group of tumours is represented to an amount of 16-25% in the main regions (57% in the women regions). This makes it impossible to compare the distribution of the three main types of MM with that of other investigations which do not include unclassifiable tumours (*Clark et al.* 1969 and *McGovern* 1970).

TABLE 3 *Primary Malignant Melanomas in Clinical Stage I*

Type	No of cases	Per cent of total
Lentigo maligna melanoma (LMM)	86	13
Superficial spreading malignant melanoma (SSMM)	259	39
Nodular malignant melanoma (NMM)	194	29
Unclassified malignant melanoma (UMM)	130	19
All primary malignant melanoma (PMM)	669	100

TABLE 4 Sex and Age at Diagnosis According to the Different Types of Malignant Melanoma

Type	Sex	0-9 No.	10-19 No.	20-29 No.	30-39 No.	40-49 No.	50-59 No.	60-69 No.	70-79 No.	80-89 No.	90-99 No.	No.	Total % of each sex
MM	M			1	3	6	4	3	7	2		26	30.2
	F			1	7	10	11	13	14	4		60	69.8
JMM	M		2	9	21	22	14	11	8	3		90	34.7
	F			11	48	39	37	15	14	4	1	169	65.3
JMM	M	3	6	3	9	14	18	15	11	6	2	87	44.8
	F		3	15	14	18	13	18	14	11	1	107	55.2
JMM	M		2	3	7	11	11	8	6	5	1	54	41.5
	F		2	5	10	21	13	11	8	4	2	76	58.5
Total	M	3	10	16	40	53	47	37	32	16	3	256	38.3
	%	1.1	3.9	6.2	15.6	20.7	18.3	14.4	11.5	6.2	1.1	100.0	
	F		5	32	79	88	74	57	50	23	4	413	61.7
	%		1.2	7.8	19.1	21.4	18.0	13.8	12.1	5.6	1.0	100.0	
Total	No.	3	15	48	119	141	121	94	82	39	7	669	100.0
	%	0.4	2.2	7.2	17.8	21.2	18.1	14.0	11.3	5.8	1.0	100.0	

Survival

Of the 669 patients in clinical stage I 429 were followed for 5 years and 184 (27.5%) for 10 years. As relatively few patients were followed any further we have ended our survival curves at 10 years. The observed, expected and specific cumulative survival rates for the 669 cases in clinical stage

I are illustrated in Fig. 9. The observed cumulative survival rate is the proportion of patients alive at a specific time after diagnosis according to the actuarial or life table method. The expected cumulative survival rate is the proportion of patients alive at a specified time after diagnosis, provided they have not been suffering from MM. The specific

TABLE 5 Distribution of the Cutaneous Malignant Melanomas Stage I

Region	All		Men		Women	
	No.	%	No.	%	No.	%
Head	164	25	70	27	94	22.5
Trunk and neck	210	31	120	47	90	22
Upper extremity	79	12	21	8	58	14
Foot	48	7	15	6	33	8
Lower extremity	161	24	25	10	136	33
Other + multiple	7	1	5	2	2	0.5
Total	669	100	256	100	413	100

TABLE 6 *Distribution of Sites According to Each Histological Type*

Site	LMM		SMM		NMM		UMM		TOTAL	
	No.	%	No.	%	No.	%	No.	%	No.	%
Head	49	57	26	10	53	27	36	21	164	25
Trunk and neck	11	21	91	35	66	34	35	27	210	31
Upper extremity	6	7	30	12	23	12	20	15	79	12
Lower extremity	11	13	86	33	39	20	25	19	161	24
Foot	2	2	24	9	12	6	10	8	48	7
Other + unknown and multiple	0	0	2	1	1	1	4	3	7	1
Total	86	100	259	100	194	100	130	100	669	100

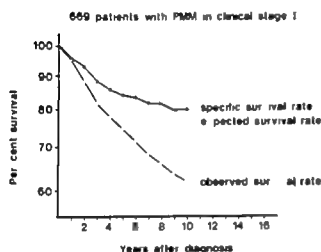


Fig 9 Survival curves illustrating the expected, the observed and the specific cumulative survival rates, respectively at each year up to 10 years after diagnosis

(relative) cumulative survival rate (R) is the ratio between the observed (O) and the expected (E) cumulative survival rate

$$(R = \frac{O}{E})$$

The cure rate is the stabilized (constant) value of the specific survival rate after a certain follow-up period (Cancer Registry of Norway 1975)

The specific cumulative survival rates of the different types of MM in our classification are illustrated in Fig. 10. In clinical stage I the 5-year specific survival rate is $98.3 \pm 2.3\%$ for LMM, $86.2 \pm 2.8\%$ for SMM, $69.5 \pm 4.2\%$ for NMM.

\pm two standard deviations

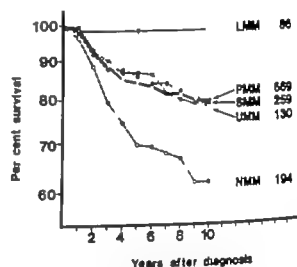


Fig 10 Survival curves illustrating the specific cumulative survival rates of the total series (PMM) and of each histological type of melanoma

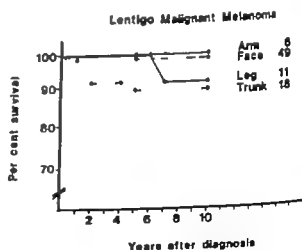


Fig 11 Survival curves illustrating the specific cumulative survival rates of 84 of the 86 lentigo malignant melanomas according to each anatomical region (Two cases on the foot are not illustrated).

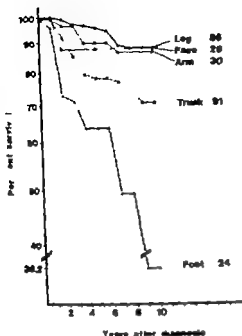


Fig 1 Survival curves illustrating the specific cumulative survival rates of 257 of the 259 superficial spreading malignant melanomas according to each anatomical region (Two cases located to other regions are not illustrated)

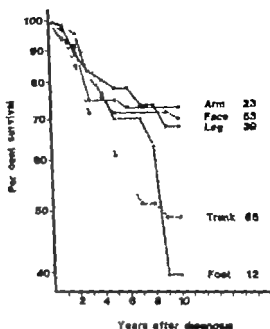


Fig 13 Survival curves illustrating the specific cumulative survival rates of 193 of the 194 nodular malignant melanomas according to each anatomical region (One case located to one of the other regions is not illustrated)

and $81.3 \pm 4.4\%$ for USM. The 10-year specific survival rate is $98.1 \pm 2.3\%$ for LMM, $78.6 \pm 4.0\%$ for SVM, $61.3 \pm 6.0\%$ for NMM and $76.7 \pm 6.6\%$ for USM. The difference in the survival rates of the 3 main types is significant at both 5 and 10 years after diagnosis. The overall 10-year specific survival rate is 79.3%.

Each of Fig 11, 12 and 13 illustrates the survival of patients with one particular type of MM according to the different regions; the overall impression is that the survival curves of MM on the arm, face and leg lie relatively close to one another and higher than that of patients with MM on the trunk. Further patients with SVM on the foot have a much worse prognosis than those with the same tumour on the trunk. NMM on the foot also has a somewhat worse prognosis than on the trunk at 10 years after diagnosis. 2 cases of LMM on the foot were, however, still alive after 12 years (not illustrated).

DISCUSSION

The reliability of the selection

We eliminated nearly 500 cases for different reasons before arriving at the 825 specimens which we considered to be histologically proven PAM (Table 2). However, there is still the problem whether the accepted sections really are representative of the respective tumours.

The surrounding epidermis had to be present at least on one side before we made a histologic classification. We know now that some SVM may have an adjacent preinvasive area including several rete ridges to one side while this area may be completely lacking to the other side. By insufficient sectioning or by the investigation of only one of the several sections such tumour may be wrongly classified as NMM. The opposite mistake is impossible. We cannot exclude the possibility that a few cases of SVM have been classified as NMM.

i.e. cases in which the surrounding epidermis was only present to one side showing no adjacent preinvasive area.

It is interesting that even though our series includes 319 cases originally registered as LM we found only 125 cases of LM and LMM. Approximately the same amount we found to be early cases of one of the 3 other types of MM and most of the rest was probably registered by us as benign (junctional) naevi.

We may have classified some cases as LMM which other might look upon as noninvasive LM. We often found it difficult to decide whether an early invasion of the papillary dermis was present or not.

Vice versa some LMM could have been classified as noninvasive LM because the early invasion even when it is multifocal is usually limited and often asymmetrical and so may have been lost when only a single section of the tumour was present.

The relatively large number of unclassifiable tumours is probably mostly due to the limitations of a retrospective survey.

The Frequency of the 3 Tumour Types.

As mentioned earlier our cases are selected, not random. However the distribution of sex, age and localization in our series (Table 4 and 5) is almost the same like that in Magnus's series. It is therefore possible that the frequency of the 3 tumour types in

our series (Table 3) indicates that in the total population of Norwegian MM. We do not find it advisable, however, to compare the frequency in our series with that in other investigations. Besides, one cannot expect the incidence of the 3 types of MM to be the same all over the world.

Difficulties in Histological Classification

The main problems of classification turned out first of all to be the distinction between LMM and SMM and secondly between SMM and NMM. Seldam *et al.* (1974) and Levene (1976) seem to have had the same difficulties.

The presence of a few isolated tumour cells in the middle of the epidermis in some LMM and the junctional proliferation of tumour cells into the hair follicles in some SMM made it difficult in several cases to distinguish between the two types. In these cases we looked for polymorphism and intraepidermal nests of spindle cells which favoured LMM whereas a monomorphous picture with intraepidermal epithelioid cells favoured SMM. In some of these cases we were not able to distinguish between LMM or SMM and used the term 'unclassifiable'. This was partly due to only a single available section. Clark had the same difficulties in 10% of the tumours when only 1-2 sections were examined. However he still had problems in 3% of the tumours when at least 5 sections were examined (Clark *et al.* 1975). Our experience leads us to believe that borderline cases between LMM and SMM may in fact, exist. According to the diastatic histogenetic theory of Mishima (1967), with which we originally agreed (Jensen *et al.* 1975), this should be impossible. The fluorescence microscope findings of Paul & Illig (1976) suggest, however, that they can occur.

Most of the problems of differentiating SMM from NMM were in cases with a sparse and often asymmetrical adjacent preinvasive area showing the involvement of 4-5 rete ridges on one side and 0-3 on the other side. If Pagetoid intraepidermal growth of tumour cells could not be seen we classified the tumour as NMM despite the involvement of 4 rete ridges adjacent to one side of the invasive tumour. If however there was indisputable Pagetoid intraepidermal growth of malignant cells, the tumour was classified as SMM. Disputable cases we termed unclassifiable. We had the impression here, also, that there are borderline cases between SMM and NMM. In some support of this is the finding that the survival curve of 58 cases with the asymmetric adjacent preinvasive area discussed above lies between the curves of SMM and NMM (Fig. 14). The number of cases is small and the curve lies closest to that of SMM but does not touch

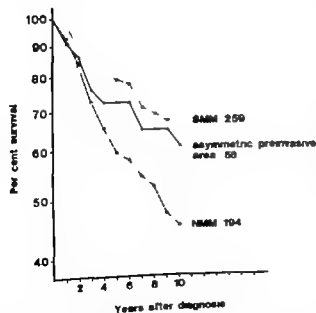


Fig. 14 Survival curves illustrating the observed cumulative survival rates of the superficial spreading malignant melanomas, the nodular malignant melanomas and the 58 cases with an asymmetric preinvasive area.

TABLE 7 Survival of the Three Types of Malignant Melanoma in Different Geographical Regions

5-year survival

Reference	Region	Year of reg.	Total no	%LMM	%SSMM	%NMM	%UMM	LMM	SSMM	NMM
Herrmann et al. 1976	Erlangen	1967-1974	139	12	35	53	0	13	66	51
McGovern 1970	New South Wales	1958-1963	202	10	42	48	0	80	70	53
Clark et al. 1969	Boston	1958-1965	209	14	54	32	0			
Present series	Norway	1955-1970	825	11	36	34	19	87	77	50

Observed death rate

it. The difference in survival is not statistically significant, but nevertheless could support the view that the greater the epidermal affinity the better is the prognosis.

When we started the investigation we were unconvinced to Clark's histological classification. At first we found it difficult to use. However as the study went on full agreement on the classification became more common. We became convinced that Clark's 3 main types really do exist, although many tumours seemed to be borderline cases between LMM and SSMM and between SSMM and NMM, respectively. However several of these cases were classifiable according to Clark's system even with our limited number of sections.

Having now used the classification some years in daily routine work, it is our view that only few melanomas are really undecidable when several sections are available from each case. In fact, the big problem is often not the classification of a melanoma but the question whether a melanotic tumour is malignant or benign.

We have sent 104 of the cases to Dr J. Liddle, Director of pathology Princess Alexandra Hospital, Brisbane Queensland, for control of the classification. The results of this will be published later.

Distribution of Age and Localisation of the 3 Tumour Types

Both Clark et al. (1969) and McGovern (1970) found a greater proportion of LMM in patients over 50 years of age than we did (Table 4). Clark also found most cases of SSMM in patients over 50 years, which was contrary to our findings and, also, those of McGovern. In addition, McGovern's series showed that most cases of NMM occurred among patients under 50 years of age while Clark found an almost similar distribution of this type in the two age groups. Both findings are in contrast to the domination of NMM among older patients in our series.

The regional distribution of our 669 cases of MM in clinical stage I (Table 6) is practically the same as that of all 825 MM (not illustrated). When the sites of our MM clinical stage I are compared with those in the unstaged series of Clark (1969) and of McGovern (1970), we find that the regional distribution of LMM is almost the same. Both Clark and McGovern found that SSMM is almost equally frequent on the head and the legs. NMM is found equally often on the head and the legs in the series of McGovern, while the findings of Clark et al. are similar to ours.

These differences mentioned above may be due to the fact that different populations have been

TABLE 8. Depth of Invasion of Each Histological Type

Type	Intraepidermal	Dermis					Sum
		Papillary	Interface	Reticular	Subcutis	?	
LMM		71%	22%	6%			
SSMM	1%	25%	42%	26%		1%	100%
NMM		1%	35%	42%	2%	4%	100%
UMM	0.5%	11.5%	36%	27%	15%	7%	100%
					5%	20%	100%

examined. The method of selection of our cases also differs from that of the other two investigations. Further the fact that we did not know anything about the age of the patient, has probably reduced our possibility to distinguish between a LMM and a SMM and between a NMM and a juvenile melanoma. This will be discussed in a later paper

Survival

In agreement with other investigations (Hette 1972 and Storck & Ott 1976) our survival curves show that it is important to follow the patients for 10 years rather than 5 to obtain a correct picture of their fate. 184 (27.5%) of our patients were still alive at 10 years after diagnosis. After 10 years the curves illustrating the specific cumulative survival rates continue almost horizontally indicating the various cure rates. In Fig. 9 the specific survival of the patients in clinical stage I becomes better than the expected survival after 9 years. It is tempting to imagine that this illustrates that long-term survivors have a better immune response than short term survivors (Kokoschka & Niebauer 1976) perhaps even better than the general population.

Table 7 shows the 5 year observed survival rates of the different types of all our 825 PMM together with those of other investigations using the same classification. There is good correspondence in the survival rates of the 3 tumour types in the different series. The prognosis of the Norwegian patients with LMM and SMM is relatively good. This may be due to the fact that the invasive growth of these MM was rather limited (early diagnosis?) (Table 8) but it may also be due to different immune response of the patients or to different types of treatment. All we know about treatment is, however, that in the total series of Magnus (1977) 98.7% were treated «surgically» while only a «biopsy» was taken from the rest of the patients (more than half of whom were over 80 years old).

A lack of correlation between survival and a particular localization of MM has been reported by some authors (Allen & Spitz 1953 Huvois *et al* 1974 Elias *et al* 1977 and Palkanen 1977b). A definite correlation has, however, been reported by other investigators (Petersen *et al* 1962, Jones *et al* 1968 McLeod *et al* 1971 Ericsson *et al* 1976 and Storck & Ott 1976). According to the «prognostic score sheet» devised by Cochran (1968) and in the modification of this system by Hardmeter (1970) MM on the head, neck and upper limbs have the best prognosis and those on the trunk and in the anogenital region have the worst. These findings and the scoring system is generally in agreement with the regional variations of prognosis in our series (Fig. 11-13). Only one of the investigations

mentioned above (Petersen *et al* 1962) has especially stressed the bad prognosis of MM on the foot. However these differences in survival of patients with MM at various skin regions become smaller when age- and sex-standardized survival rates are used (Magnus 1977).

CONCLUSIONS

To the questions we asked ourselves at the beginning of this investigation we are now able to give the following answers.

A. We are able to classify 81% of the sections we have examined and 19% were left inclassifiable. This latter group is large, partly because of difficult differential diagnostic problems, partly because of unrepresentative sections or sections of poor technical standard.

B. The essential problems turned out to be the difference first of all between LMM and SMM and secondly between SMM and NMM. We think it is probable that borderline cases really do exist. Having used the classification in our daily routine work since we started our retrospective investigation 4 years ago it is, however, our experience that only a few tumours are really unclassifiable provided that several sections are available from each case.

C. The incidence of the 3 types of melanoma in our study: LMM 13% SMM 31% NMM 29% and UMM 19%. Because of the method of selection it is not possible to say whether this reflects the incidence in the total population of Norwegian MM.

D. In clinical stage I the 10-year specific cumulative survival rate (~ the cure rate) is 98.3% of LMM 78.6% of SMM and 61.3% of NMM. The 5- and 10-year specific cumulative survival rates are significantly different for the 3 main types of MM. The overall specific cumulative survival rate is 79.3%. The prognosis of the 3 types of MM in our series seems to vary much in the same way as in other investigations and, concerning LMM and SMM, appears to be better. The prognosis of the most serious type of MM viz. NMM is not at all hopeless in Norway when the cure rate is about 60%.

Since the prognosis varies according to the histological type pathologists should no longer only use the term «malignant melanoma», but should try to classify the tumour into lentigo maligna melanoma, superficial spreading melanoma or nodular melanoma.

We wish thank Professor O. H. Iversen, Institute of Pathology Rikshospitalet, Oslo and Dr. K. Magnus, Cancer Registry of Norway Oslo, for help and advice.

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THE CLASSIFICATION OF PRIMARY CUTANEOUS MALIGNANT MELANOMA

A Prospective Study of 60 Cases Using Clark's Classification

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Larsen, T. E.: The classification of primary cutaneous malignant melanoma. A prospective study of 60 cases using Clark's classification. *Acta path. microbiol. scand. Sect. A*, 86: 451-459 1978.

A series of 60 primary cutaneous malignant melanomas has been studied by serial block technique. The resulting 492 sections have been classified as junctional naevus with or without atypia and preinvasive or invasive malignant melanoma according to Clark (1967). No sections showed lentigo maligna (acrolentiginosa). The overall classification resulted in 49 superficial spreading malignant melanomas, 6 nodular malignant melanomas and 5 undeclassifiable malignant melanomas. In 3 cases (5%) there was inconsistency between the classification of the central section and the overall classification of the tumour. Five theoretical growth patterns have been postulated ranging from that of the pure superficial spreading malignant melanoma completely surrounded by a preinvasive area to the pure nodular malignant melanoma which completely lacks any such area. Borderline cases between these two types certainly seem to exist. Features such as morphological pagetoid growth of tumour cells, co-existence of a benign melanocytic component and histological changes indicating tumour regression have been discussed. It is recommended that at least 3 major blocks should be taken from all malignant melanomas up to 25 mm in diameter and more if the tumour is larger.

Key words: Melanoma, classification, growth patterns.

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Received 2 iv 78 Accepted 5 vi 78

In 1967 Clark and in 1969 Clark *et al.* proposed a histological classification of primary cutaneous malignant melanoma (MIM). This included 3 types, lentigo maligna melanoma (LM), superficial spreading malignant melanoma (SSM) and nodular malignant melanoma (NM). These three types have respectively a good, an intermediate and a bad prognosis which is also dependent upon the level of invasion.

In 1967 Clark said that in the present feeling that the consistent and accurate classification of melanoma is dependent upon careful gross-microscopic correlation, including multiple sections of the primary. Later Clark *et al.* 1975 stated that it was impossible to distinguish between LM and SSM in at least 10% of the cases if only one or two

sections were available, and that this figure was reduced to 3% when 5 sections were available.

McGowen (1972) found that where is some degree of overlap between the three forms of melanoma so that occasionally there may be some difficulty in both the clinical and histological categorizations.

Larsen & Grude (1978) made a retrospective histological study of 669 cases of MIM and found an overlap between LM and SSM and also between SSM and NM. They found many (19%) MIM which were undeclassifiable. It was concluded that this was probably due to the fact that only a limited number (1-3) from each case had been examined.

A prospective study of MIM by serial block technique has therefore been made in an attempt to answer the following questions:

- A. Do overlaps between the three types of MM really exist?
- B. Is the histological picture of a particular type the same in all areas, so that classification can be safely based on one or a few sections?
- C. What is the best routine method of taking sections in relation to the macroscopic appearance so as to obtain an optimal histological classification?

SERIES

During 1976 and 1977 60 MM were collected at Laboratorium for pathologi A/S Oslo and at the Department of Pathology the Rikshospital Oslo (58 and 2 cases, respectively).

The 60 specimen came from 59 patients (34 men and 25 women) aged 18 to 85 years (mean age 49 years). No patients have been followed for more than 1½ year.

METHOD

One thin (max. 2 mm) tissue block was taken from the centre of each tumour and sections were classified histologically according to Clark's system. (Clark 1967). The remainder was mounted on cardboard and photographed. A drawing in natural size was also made with an indication of the position of the inkling block, the surface formation and variations of colour. The largest diameter of each tumour was 4-40 mm (mean 16 mm). In each case a series of 3-18 (median 8) tissue slices (each 2 mm thick) was cut. The slices were embedded in paraffin separately and given consecutive numbers. One to three sections of each block was stained with hematoxylin-eosin-saffron.

The cases were examined histologically as they were received at the laboratories. Totally 492 sections included a classifiable neoplastic proliferation of melanocytes. This was classified under one of the following headings.

Junctional naevus i.e. more or less regular proliferation of junctional melanocytes.

Junctional naevus with atypia i.e. continuous or interrupted junctional proliferation of atypical melanocytes with epidermal hyperplasia and with or without solar elastosis in the dermis.

Lentigo maligna i.e. continuous junctional proliferation of atypical melanocytes with epidermal atrophy (Uthak Petzold 1974) and with dermal solar elastosis.

non-invasive SVM i.e. a junctional naevus with atypia and with in addition intrapapillary growth of atypical melanocytes (apagoid growth).

LMM i.e. as in lentigo maligna but with additional dermal invasion.

SMM i.e. as in non-invasive SVM but with additional dermal invasion. The non-invasive area affects more than 3 rete ridges.

NMM i.e. an invasive MM with no or a very narrow adjacent non-invasive area affecting not more than 3 rete ridges.

UAM i.e. an invasive MM with an undetectable adjacent non-invasive area affecting more than 3 rete ridges, or an adjacent non-invasive area of superficial spreading type affecting more than 3 rete ridges but with in addition dermal changes underneath indicating clinical tumour regression or the possible co-existence of a benign naevus complicating the classification.

not possible to judge i.e. poor technical quality or an insufficient representation of the adjacent epidermis making the quality and the quantity of a non-invasive area uncertain.

According to the various combinations of these histological pictures 5 theoretical growth patterns of SVM and NMM have been proposed (Table 1).

growth pattern A A SVM completely surrounded by a non-invasive area.

growth pattern B A SVM surrounded by a non-invasive area to an extent of 75% of the circumference.

growth pattern C A SVM surrounded by a non-invasive area to an extent of 50% of the circumference.

growth pattern D A SVM surrounded by a non-invasive area to an extent of only 25% of the circumference.

growth pattern E A NMM with the complete lack of a non-invasive area.

RESULTS

Table 1 shows the overall classification of each case according to the dominant histological picture. It is seen that for the most sections were classified as

TABLE 1 Number of Cases of Each Tumour Type and Histological Diagnosis of Each Tissue Block

The overall diagnosis of each tumour		The histological diagnosis of each tissue block						Total No of tissue blocks
Tumour type	No.	Junctional naevus No	Junctional naevus with atypia No.	Non-invasive SVM No	SVM No	NMM No	UAM No	
SVM	49	6	20	87	253	37	7	410
NMM	6		1	3		47		47
UAM	5	5				8	20	35
Total No	60	11	21	90	253	90	27	492

SMM. No single section was classified as lentigo maligna and accordingly was no case classified as LMN.

The Relationship between the Various Histological Pictures in Each Case Table 2 illustrates the various combinations of histological pictures among the classifiable cases. In 31 cases all sections showed growth pattern A. In 6 cases all sections showed growth pattern E. Accordingly the overall classification of these cases was SMM and NMM, respectively. Between these two extreme pictures

there was, however, a wide spectrum of combined histological changes in 18 cases of SMM. These cases showed an asymmetrical adjacent non-invasive area of a varying extension corresponding to growth pattern B-D.

Pagetoid Intraepidermal Growth of Tumour Cells Related to the Classification of the Single Section A satisfactory gradation of Pagetoid growth was found to be impossible. The overall impression was, however, that most SMM-sections showed an extended and considerable intraepidermal Pagetoid

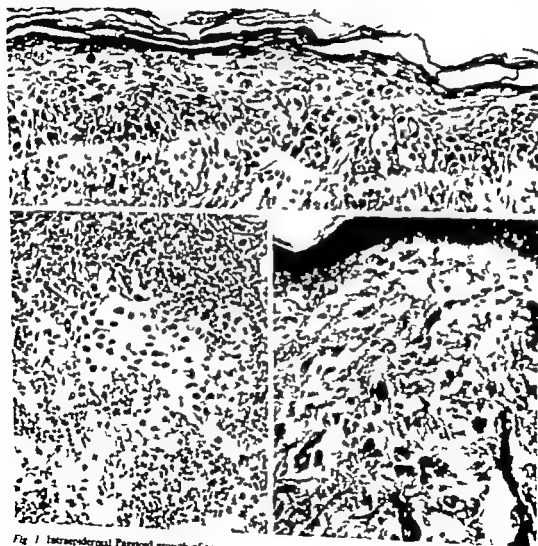


Fig. 1 Intraepidermal Pagetoid growth of tumour cells in a SMM. $\times 195$

Fig. 2 A dense lymphocyte infiltration which surrounds and invades tumour tissue in the papillary dermis splitting off single tumour cells which often look degenerated. $\times 195$

Fig. 3 An area in the staddle of a SMM showing the lack of tumour tissue. There is a moderate vascular proliferation and a slight lymphocyte infiltration in the papillary dermis. $\times 195$

- A. Do overlaps between the three types of MM really exist?
- B. Is the histological picture of a particular type the same in all areas so that classification can be safely based on one or a few sections?
- C. What is the best routine method of taking sections in relation to the macroscopic appearance so as to obtain an optimal histological classification?

SERIES

During 1976 and 1977 60 MM were collected at Laboratorium for patologi A/S Oslo and at the Department of Pathology the Rikshospital, Oslo (58 and 2 cases, respectively).

The 60 specimen came from 58 patients (34 men and 24 women) aged 18 to 83 years (mean age 49 years). No patients have been followed for more than 1½ year.

METHOD

One thin (max. 2 mm) tissue block was taken from the centre of each tumour and sections were classified histologically according to Clark's system (Clark 1967). The remainder was mounted on cardboard and photographed. A drawing in natural size was also made with an indication of the position of the missing block, the surface formation and variations of colour. The largest diameter of each tumour was 4–40 mm (mean 16 mm). In each case a series of 3–18 (medium 8) tissue slices (each 2 mm thick) was cut. The slices were embedded in paraffin, separately and given consecutive numbers. One to three sections of each block was stained with hematoxylin-eosin-saffron.

The cases were examined histologically as they were received at the laboratories. Totally 492 sections included a classifiable neoplastic proliferation of melanocytes. This was classified under one of the following headings.

junctional naevus, i.e. more or less regular proliferation of junctional melanocytes

junctional naevus with atypia i.e. continuous or interrupted junctional proliferation of atypical melanocytes with epidermal hyperplasia and with or without solar elastosis in the dermis.

lentigo maligna i.e. continuous junctional proliferation of atypical melanocytes with epidermal atypia (Kuhnl Petzold 1974) and with dermal solar elastosis.

non-invasive SMM i.e. a junctional naevus with atypia and with in addition intraepidermal growth of atypical melanocytes (»Pagetoid growth»).

LM i.e. as in lentigo maligna but with additional dermal invasion.

SMM i.e. as in non-invasive SMM but with additional dermal invasion. The non-invasive area affects more than 3 rete ridges.

NMM i.e. an invasive MM with no or a very narrow adjacent non-invasive area affecting not more than 3 rete ridges.

U/M i.e. an invasive MM with an undecipherable adjacent non-invasive area affecting more than 3 rete ridges, or an adjacent non-invasive area of superficial spreading type affecting more than 3 rete ridges but with in addition dermal changes underneath indicating clonal tumour regression or the possible co-existence of a benign naevus complicating the classification.

not possible to judge i.e. poor technical quality or an insufficient representation of the adjacent epidermis making the quality and the quantity of a non-invasive area uncertain.

According to the various combinations of these histological pictures 5 theoretical growth patterns of SMM and NMM have been proposed (Table 2).

growth pattern A A SMM completely surrounded by a non-invasive area.

growth pattern B A SMM surrounded by a non-invasive area to an extent of 75% of the circumference.

growth pattern C A SMM surrounded by a non-invasive area to an extent of 50% of the circumference.

growth pattern D A SMM surrounded by a non-invasive area to an extent of only 25% of the circumference.

growth pattern E A NMM with the complete lack of a non-invasive area.

RESULTS

Table 1 shows the overall classification of each case according to the dominant histological picture. It is seen that far the most sections were classified as

TABLE 1 Number of Cases of Each Tumour Type and Histological Diagnosis of Each Tissue Block

The overall diagnosis of each tumour		The histological diagnosis of each tissue block						Total No of tissue blocks
Tumour type	No	Junctional naevus No	Junctional naevus with atypia No	Non-invasive SMM No	SMM No	NMM No	U/M No	
SMM	49	6	20	87	253	37	7	410
NMM	6					47		47
U/M	5	5	1	3		6	20	35
Total No.	60	11	21	90	253	90	27	492

Fig 4 A typical SVM with a multicoloured surface, but only a very narrow infiltrated, pigmented halo. Histologically growth pattern A. Orig. diam. 3.8 cm

Fig 5 A less typical, hemispherical SVM with a uniformly coloured surface. A very narrow uninfiltated, pigmented halo is present to one side (arrow). Histologically growth pattern C. Orig. diam. 4 cm

Fig 6 A NVM with a light brown infiltrated halo to one side of the dark brown tumour (arrow). Histologically growth pattern B. Orig. diam. 2.5 cm.

growth (Fig 1), while this phenomenon was completely lacking in most of the NVM-sections. In some SVM-sections it was, however rather sparse and limited.

Histological Changes in Areas Corresponding to Clinical (Macroscopical) Tumour Regression. Changes indicating tumour regression (McGovern 1976) were found in 4 cases of SVM (men aged 43-71 years) and in 3 other cases (men aged 45-71 years) in which classification was made impossible by these changes. Histologically there were some areas with a heavy lymphocyte infiltration surrounding and splitting up intradermal collections of tumour cells (Fig. 2). One might also find below the epidermal-dermal junction small collections of Civatte bodies (which could be dead melanocytes as well as dead keratinocytes) and in addition a varying lymphocyte infiltrate. In areas where the tumour tissue had completely disappeared there was a somewhat fibrotic, richly vascularized collagen

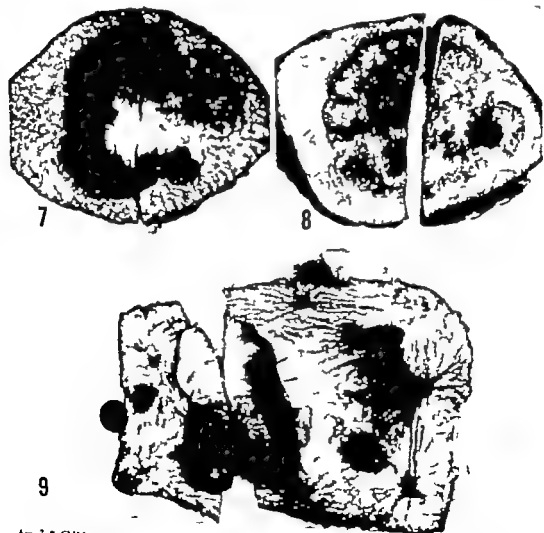
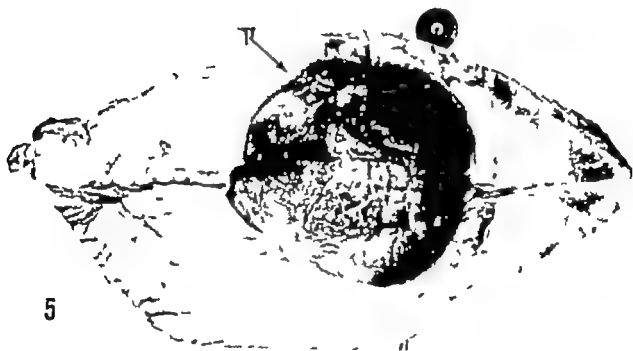


Fig 7-9 SVM including white scar-like areas (arrows) indicating clinical tumour regression. Orig. diam. 5.5, 6 and 1.5 cm, respectively









4



5



6

Theoretical growth pattern based on the distribution of histological patterns		Histological picture/diagnosis of each section						Total no. of specimens	No. of lesions	No. of pain/lesion
		Benignive lesions	SMM	MM	LM	NMM				
	A	83	181					274	21	
	B	10	14	18				42	6	
	C	9	8	8		5		30	4	49 SMM
			5	17	3	10		35	4	
	D	(1)		2	4	22		30	4	
	E					47		47	6	6 NMM
Total		113	208	45	7	84		457	55	

*Including junctional naevi + atypia and progressive SMM

The presence of borderline cases between the different types of MM. The classification of MM with growth patterns A-B (Table 2) as SMM and of those with growth pattern E as NMM seems logical. I find it, however, meaningless to classify a tumour as SMM if it includes only a very limited non-invasive area corresponding to 25% or less of the circumference (growth pattern D), unless a heavy intraepidermal pagetoid growth of tumour cells is present and there are changes around the tumour indicating regression of an originally more extended non-invasive area. The tumours with growth pattern C are the real borderline cases between SMM and NMM. The study of a much larger series with a long follow-up of the patients might reveal whether one of the classification of MM with growth pattern C and D should first of all pay attention to the NMM-like part of the tumour. This might turn out to be responsible for the survival of the patient by showing the poorest epidermal affinity and probably therefore the lowest degree of differentiation.

The absence of LM in the present series is in agreement with the experience of other histopathological laboratories that the incidence of LM is very low in Norway. It may be comparable with 4.9% found in USA 1976 (Kopf *et al.* 1977), but may be even lower. No borderline cases between

LM and SMM were found as no single section was classified as lentigo maligna or LM. One SMM included several sections showing a limited lentigo maligna-like area. No solar elastosis was, however, present, and there were dermal changes corresponding to those illustrated in Fig. 3 indicating clinical tumour regression which might be responsible for the atrophic epidermis. According to the dualistic theory of histogenesis claimed by Melnikow (1967) such borderline cases should not exist. The findings of Paul & Joly (1976) indicate, however, that they can exist. A much larger series has to be studied by serial block technique in order to decide whether LM and SMM really are two different tumour types or only two manifestations of the same tumour appearing in a young well-preserved and an old atrophic skin, respectively.

Gross-examination and blocking. The variable growth pattern in SMM and NMM stresses the importance of taking sections in a way which will demonstrate each particular pattern.

Fig. 10 illustrates two ways of taking tissue blocks from a MM with a diameter of max. 25 mm. a) 3 cross sections and b) 1 cross section and 2 sections at a right angle with this. These two methods seems to be equally effective. Evidence of the limited lack of an adjacent non-invasive area in growth pattern B is easily lost by both methods.

tissue subepidermally which might still show some edema and lymphocytes (Fig. 3)

The Co-Existence of MM with a Benign Intradermal Component This was found in 4 cases of SMM. Further in 2 cases of UMM this phenomenon was the sole cause of the difficult classification. The benign cells were found underneath the malignant invasive tumour or to one side of it.

Comparison of the Central Primary Section with the Serial Sections. One of the primary sections was considered not possible to judge. A real inconsistency was found in 3 cases (5.1%).

Comparison of the Macroscopical Appearance of the Tumour with the Histological findings In most cases it was rather easy to classify the tumour already from its macroscopical appearance. Even the slightest sign of an unpalpable, pigmented halo around the central elevated part of the tumour corresponded well with the overall classification as SMM (Fig. 4 and 5). The apparently lack of such a halo did not, however exclude this diagnosis, as a narrow asymmetrical pre-invasive area could often not be seen macroscopically. The surface of the NMM was mostly uniformly coloured, and an uninfiltreated pigmented halo was never present (Fig. 6). White, more or less scar like areas indicating tumour regression were found among SMM only (Fig. 7-9). The co-existence with a benign intradermal component could not be recognized macroscopically.

DISCUSSION

Because of the short observation of the patients no conclusion can be made about their survival. Prognostic problems will therefore not be discussed.

The relationship between the non-invasive and the invasive part of a MM The present series has given the opportunity to investigate only SMM and NMM.

The variable extension of a non-invasive area in this series corresponds with the different patterns of «field changes» found by Cochran 1968. Further Cochran describes how «there is a gradual increase in melanocyte incidence from that of normal for the area in which the tumour is situated until first lentigo and then junctional activity is seen as the tumour is approached.» This is also in agreement with the findings in the present series. Clark *et al* 1975 claim that the non-invasive area precedes the invasive one. This seems logical but cannot be proven by a histological study like the present one.

Intraepidermal Pagetoid growth of tumour cells It was found safer to base the classification of the single sections as well as the total tumour on the extension of the non-invasive area than on the grade

of Pagetoid growth. There was, however one exception from the rule: Classification as SMM was preferred when the non-invasive area was narrow including no more than 3-(4) rete ridges, but an extensive intraepidermal Pagetoid growth of tumour cells in this area and superficial to the invasive part of the tumour was present.

Histological changes corresponding to clinical (macroscopical) tumour regression. These changes were found in the centre of the tumour or at the periphery. In the latter situation a junctional proliferation of melanocytes was sometimes found outside this area. Such a finding may lead to confusion as to whether two separate foci exist side by side, or whether all changes belong to one tumour. The latter explanation seems the most acceptable in the cases illustrated in Fig. 8 and 9. Histological changes corresponding to clinical (macroscopical) tumour regression may also lead to misdiagnosis of SMM as NMM or vice versa because of destruction of either an adjacent intraepidermal area or a dermal MM component underneath an affected epidermis. It is therefore important to be aware of these changes.

Such changes were found in 7 out of 60 cases (11.7%). This corresponds to the findings of Little (1972). The figures should, however not be taken too seriously as both series are rather small.

The co-existence of a benign melanocytic component Evidence of benign proliferation of melanocytes was found in the non-invasive area in 5 SMM and in 3 UMM. None of these cases had a benign dermal component. This was, however found in 6 other cases (4 SMM and 2 UMM). The total incidence of a co-existent benign component is 11 of 60 cases (18.3%), while the incidence of a co-existent intraepidermal benign component is 6 of 60 cases (10%). This latter figure is in agreement with the findings of Clark *et al* (1969) while Cochran (1968) and McGovern (1970) found a benign intraepidermal component in 72% and 30% of the cases, respectively. These high figures may be due to an overestimation of small, slightly atypical MM cells as benign, proliferating melanocytes (Little 1972, Reed *et al* 1975).

There has been much discussion in the literature as to whether a MM may develop from a benign melanocytic tumour the naevus (Allen & Spitz 1953, Cochran 1968, Clark *et al* 1969, Clark *et al* 1975, McGovern 1972). However neither the clinical information about a pre-existent benign lesion nor the histological finding of a co-existent benign component proves anything about histogenesis. The two tumours can have developed from normal melanocytes in the same area at the same time or subsequently.

Treatment groups/patient based on the histological picture		Histological picture/diagnosis of each section					Total no. of sections	No. of tumours	No. of borderline cases
		Non-invasive area	SSIM	LMM	NMIM				
	A	83	181				274	31	
	B	10	14	18			42	8	
	C	9	8	8		5	30	4	49 SSIM
	D		5	17	3	10	35	4	
	E	(1)		2	4	21	28	4	
	F					47	47	6	6 NMIM
Total		113	208	45	7	84	457	56	

*Including junctional nevus + atypia and preinvasive SSIM

The presence of borderline cases between the different types of MM. The classification of MM with growth pattern A-B (Table 2) as SSIM and of those with growth pattern E as NMIM seems logical. I find it, however, meaningless to classify a tumour as SSIM if it includes only a very limited non-invasive area corresponding to 5% or less of the circumference (growth pattern D), unless a heavy intraepidermal pagetoid growth of tumour cells is present and there are changes around the tumour indicating regression of an originally more extended non-invasive area. The tumours with growth pattern C are the real borderline cases between SSIM and NMIM. The study of a much larger series with a long follow-up of the patients might reveal whether one at the classification of MM with growth pattern C and D should first of all pay attention to the NMIM-like part of the tumour. This might turn out to be responsible for the survival of the patient by showing the poorest epidermal affinity and probably therefore the lowest degree of differentiation.

The absence of LMM from the present series is in agreement with the experience of other histopathological laboratories that the incidence of LMM is very low in Norway. It may be comparable with 4.9% found in USA (Kopf *et al.* 1977), but may be even lower. No borderline cases between

LMM and SSIM were found as no single section was classified as lentigo maligna or LMIM. One SSIM included several sections showing a limited lentigo maligna-like area. No solar elastosis was, however, present, and there were dermal changes corresponding to those illustrated in Fig. 3 indicating clinical tumour regression which might be responsible for the atrophic epidermis. According to the dualistic theory of histogenesis claimed by Mahle (1967) such borderline cases should not exist. The findings of Paul & Iltis (1976) indicate, however, that they can exist. A much larger series has to be studied by serial block technique in order to decide whether LMM and SSIM really are two different tumour types or only two manifestations of the same tumour appearing in a young well-preserved and an old atrophic skin, respectively.

Gross-contamination and blocking. The variable growth pattern in SSIM and NMIM stresses the importance of taking sections in a way which will demonstrate each particular pattern.

Fig. 10 illustrates two ways of taking these blocks from a MM with a diameter of max. 25 mm: a) 3 cross sections and b) 1 cross section and 2 sections at a right angle with this. These two methods seems to be equally effective. Evidence of the limited lack of an adjacent non-invasive area in growth pattern B is easily lost by both methods.

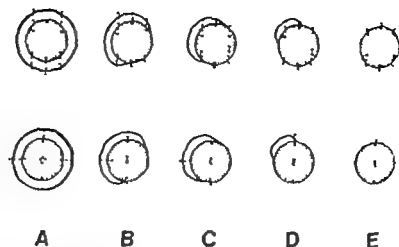


Fig 10 Two different ways of taking sections from a NMM. The representation of the various histologic pictures at the different growth patterns of SMM and NMM is illustrated

This does not matter as these cases should be classified as SMM all the same. Evidence of the presence of a small non-invasive area in growth pattern D is also easily lost by both methods. This will lead to the misinterpretation of a SMM as a NMM. In my opinion this is not very serious as those cases are likely to behave like a NMM.

If the tumour diameter is 25 mm or more, one should take more tissue blocks. In order to get the right impression of the growth pattern of a particular MM the tissue blocks should include areas with a visible, non-infiltrated, pigmented halo corresponding to a LMM or a SMM as well as areas without such a halo which usually correspond to a NMM-like picture histologically. One should avoid scar-like areas indicative of tumour regression, especially if they affect the periphery of the tumour because such changes often make tumour classification difficult.

The consistency of the classification of the central cross section with that of the whole tumour. A 5% inconsistency between the classification of the central cross section and the overall classification of the tumour seems acceptable. It must, however, be accepted with caution, because the series is small. Further, one can not exclude the possibility that the figure might have been larger if the study had been made blindly i.e. with all 492 sections mixed and studied randomly. This was not done because the cases represent part of the routine work of the laboratories to which they were sent. One did therefore not want to withhold a more correct diagnosis than the primary one based on the central section for more than one to a few weeks.

CONCLUSIONS

The present study has shown that

- There is a histological overlap between SMM and NMM due to great variation in the extension of the adjacent non-invasive area of superficial spreading type. There is no overlap between LMM and SMM. This conclusion must be accepted with caution because the series is small.
- The histological picture in the primary central cross section diverged from the final classification in 3 out of 60 cases (5%). This figure seems acceptable.
- As a routine procedure at least 3 tissue blocks should be taken from MM with a diameter up to 25 mm. Extra blocks should be taken whenever the diameter is more than 25 mm. Areas with as well as without a pigmented halo around the central tumour should be included and scar-like areas indicative of tumour regression should be avoided.

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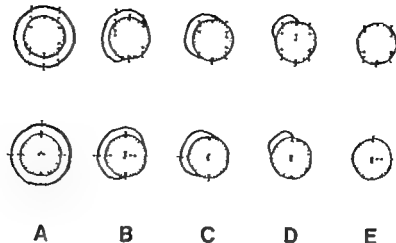


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FLOW CYTOMETRIC DNA ANALYSIS IN FINE NEEDLE ASPIRATION BIOPSIES FROM PATIENTS WITH PROSTATIC LESIONS: DIAGNOSTIC VALUE AND RELATION TO CLINICAL STAGES

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Frederiksen, P., Thomsen, P., Kyr, T. B. & Bichel, P. Flow cytometric DNA analysis in fine needle aspiration biopsies from patients with prostatic lesions. Diagnostic value and relation to clinical stages. *Acta path. microbiol. scand. Sect. A*, 86: 461-464, 1978.

Transrectal fine needle aspiration biopsy was carried out in 59 patients with cancer of the prostate, benign prostatic hypertrophy and prostatitis for cytomorphological examination and flow cytometric DNA analysis. One major single peak in the DNA histogram was observed in patients with benign hyperplasia, prostatitis and in some patients with carcinoma. A DNA histogram with a second or third peak was always compatible with carcinoma and was found both in patients with carcinoma in stage I-II and stage III-IV. It is suggested that patients with hyperplastic cancers in stage I-II could benefit from early treatment.

Key words: Flow cytometry, DNA, Prostatic carcinoma, Aspiration biopsy, Prostatic cytology, Clinical stages.

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Received 18.7.78 Accepted 1.8.78

Transrectal aspiration biopsy from the prostate as evolved by *Frantzen et al.* (1960) is a rapid and reliable method.

This technique has therefore gained increasing importance in the management of patients with prostatic lesions serving as an initial morphological diagnosis and enabling repeated control biopsies.





It is generally accepted that malignant cells have increased nuclear DNA content in contrast to diploid DNA values observed in most benign cells (*Uruckenhoffer et al.* 1954; *Bolon & Sandström* 1975).

Correspondingly *Tanases et al.* (1966), *Jacobson*

(1968), *Springer et al.* (1974) and *Zeinberg & Epstein* (1976) observed diploid DNA values in benign prostatic lesions and increased DNA values in most prostatic cancers. These findings could furthermore be confirmed using flow cytometric single cell DNA analysis (*Springer et al.* (1976), *Bichel et al.* (1977)). However, *Springer et al.* (1976) found flow cytometric analysis alone unsuitable for diagnosing prostatic carcinoma.

The purpose of this study has been to evaluate the diagnostic significance of supplementary flow cytometric DNA analysis in a cytological material obtained by fine needle aspiration biopsies from patients with prostatic lesions and furthermore to correlate the DNA content in carcinomas to the clinical stages.

DNA HISTOGRAM

					
CYTOLAGNOSTIC		ONE PEAK	ONE PEAK +	TWO PEAKS	THREE PEAKS
HYPERPLASIA	27	14	3		
PROSTATITIS	12	10	2		
CARCINOMA STAGE I-II	12	3 NO 2 NO 1	2 NO 2 NO 1	5 NO 2 NO 3	2 NO 2
CARCINOMA STAGE III-IV	18	4 NO 2 NO 3	3 NO 3	8 NO 7 NO 1	3 NO 1 NO 2
TOTAL	59	32	10	13	5

Flow Cytometric DNA Analysis in 59 Patients with Prostatic Diseases

Typical histograms are illustrated (as top) and recorded as peaks (below)

Each histogram is based on the analysis of about 50 000 cell nuclei

Ordinate indicates cells/channel or relative count, and abscissa the relative fluorescence intensity (channel number)

One peak indicates all counted cell nuclei are the diploid DNA area, one peak + an additional small fraction of less than 10 per cent tetraploid cells. Two or three peaks represent significant hyperploid cell fractions

WD = well-differentiated carcinoma

MD = moderately - differentiated carcinoma

PD = poorly - differentiated carcinoma

diploid area was the most frequent finding representing a number of different cell types i.e benign prostatic epithelial cells, inflammatory cells and carcinoma cells, especially of well-differentiated degree

Cases with a discrete tetraploid DNA peak in the histograms showed a similar cellular pattern and could not be distinguished based on cytomorphology from cases with only diploid cells. A possibility which cannot be ruled out is that the discrete second peak may prove doublets of cell nuclei, although nuclear aggregates were not found in the ethidium bromide stained suspensions before the FCM analysis.

Only cases with two or more significant peaks in the DNA histograms indicating hyperploid cells were compatible with carcinoma, and a finding of only one peak was of no diagnostic support in the interpretation of the cytological smear.

It has already been demonstrated by Espeell (1971) that prostatic carcinomas of low or differentiated degrees predominate in patients in clinical stage III-IV. In a previous study Bickel *et al* (1977) demonstrated a positive correlation between increasing DNA in tumour cells and increasing cytological anaplasia. A similar positive correlation between increasing DNA content and the clinical stage of the patient could not be demonstrated in this study as hyperploid DNA peaks were almost equally frequent findings in patients both in stage I-II and stage III-IV. The significance of these findings cannot presently be assessed.

However, in another series of long term treated patients with prostatic cancer predominance of diploid DNA content in tumour cells was only observed in patients in good clinical condition (Kjær *et al* 1978). Furthermore, in a prospective series of patients with prostatic carcinoma, a disappearance

Patients

This study comprises 59 consecutive patients from the department of urological surgery suffering from various prostatic diseases. Thirty patients had untreated carcinoma of the prostate, 17 had benign hyperplasia and 12 patients non specific prostatitis. Thirty patients with untreated carcinoma had a full clinical examination including determination of the ESR, roentgenography of the skeleton combined with bone scintigraphy, renal function tests and urography and determination of the acid and alkaline phosphatases. The patients were grouped according to the classification given by the Veterans Administration Cooperative Urological Research Group (Mellinger 1965).

Transrectal aspiration biopsies of the prostate was performed with the Franzen instrument (Franzen et al 1960). The aspirates were used for 1 FCN analysis and 2 cytomorphological examination.

Flow Cytometric Analysis

The aspirated material was washed once in TRIS-EDTA buffer pH 7.4 at 4° C. After centrifugation at 200 g for 2 minutes the cells were prepared according to the following detergent technique described by Lindelöf (1977). The cells were suspended, stained and kept at 4° C in the following solution: sodium citrate 1000 mg, ethidium bromide (BDH) 10 mg, sodium chloride 584 mg, the detergent Nonidet P40 (BDH) 300 µl, RNase (BDH) 10 mg, distilled water to make 1000 ml, pH 7.6. By this procedure cells are separated, naked nuclei obtained and stained. The RNA has been digested by RNase. After filtration through a 100 µ filter this method results in a suspension of single nuclei the purity of which was controlled under the microscope.

After renewed staining for at least 10 minutes the nuclei were analysed in a flow microfluorometer (Cytosfluorograph Biophysics Inc). The signals were sorted and stored in a DIDAC (Inter technique) multi channel analyser and presented as a plot of the number of measured cells against the relative fluorescence intensity (or relative DNA content) on a chart recorder. The calculation of the percentage distribution of the various cell populations was performed automatically by integration on the DIDAC 800. Non stimulated human lymphocytes were used as standards.

From each sample between 17 000 and 100 000 nuclei (on average 50 000) were analysed. The whole procedure including preparation, staining and measuring could be completed within 30 minutes.

Cytomorphological Examination

The aspirates were air-dried, stained according to the May-Grunwald-Giemsa method and classified according to the criteria initially suggested by Expositi (1966, 1971).

Prostatic carcinoma comprises cells with marked nuclear atypia with prominent nucleoli, decreased cytoplasmatic nuclear ratio and often a crowding of the cytoplasm into a central mass with the nuclei in a peripheral circle (microadenomatous complex formation). A further subdivision in the degree of differentia-

tion (well, moderately and poorly) is based on the degree of cellular differentiation and microadenomatous complex formation. In benign hyperplasia, epithelial aggregates, often showing honeycomb structure are found whereas in prostatitis a mixture of benign epithelial cells and inflammatory cells i.e. macrophages, neutrophil granulocytes and lymphocytes are seen.

RESULTS

The DNA analyses in the 59 patients are summarized together with the corresponding histograms in Table 1. Stages I-II with cancer confined to the gland are included in one group as are the stages III-IV with carcinoma extending beyond the gland or with metastases.

In 31 patients, one peak in the diploid area, as compared to unstimulated human lymphocytes, was observed. The simultaneously obtained smears in these patients revealed both benign and malignant cells. Fourteen specimens could cytologically be characterized as benign hyperplasia and 10 as prostatitis. Seven patients had carcinoma cells. The cancer cells were well-differentiated in 3 and moderately differentiated in 4. Column 2 in Table 1 comprises 10 patients with an additional small fractions of tetraploid cells (less than 10 per cent). The cytomorphological pattern was undistinguishable from cases with only a single diploid peak. The cancer cells were well-differentiated in 4 cases and moderately differentiated in one.

The third column in Table 1 comprises 13 patients with a second significant peak of hyperplod cells in the histogram. All the smears were cellular and contained numerous carcinoma cells. Two of the cancers were well-10 moderately and one poorly-differentiated. The least amount of hyperplod cells (13 and 17 per cent) were observed in the two well-differentiated cancers.

In the last column with two or more hyperplod peaks three cancers were moderately differentiated and two poorly differentiated.

An almost equal distribution of the DNA histograms with one or several peaks was found in the two main clinical groups whereas poorly differentiated carcinoma (3 patients) only was observed in clinical stage III-IV and well-differentiated cancers predominated in stages I-II.

DISCUSSION

In this study we have compared the cytomorphological pattern in stained smears in prostatic lesions with the DNA histograms obtained by flow cytometric analysis of the single cell DNA content. One single peak in the DNA histogram in the

PATHOLOGY OF MUCINOUS OVARIAN CYSTADENOMAS

1. Argyrophil and Argentaffin Cells and Epithelial Mucosubstances

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Klemi, P. J. Pathology of mucinous ovarian cystadenomas. I. Argyrophil and argentaffin cells and epithelial mucosubstances. Acta path. microbiol. scand. Sect. A, 86: 465-470, 1978.

The presence of argyrophil and argentaffin cells and epithelial mucosubstances was studied in 187 mucinous ovarian cystadenomas of varying malignancy. Sixty-two per cent of the benign tumours contained these cells. The corresponding figures for tumours of borderline malignancy and malignant tumours were 60 and 31, respectively. The epithelial cells of the mucinous cystadenomas characteristically contained periodic acid-Schiff reactive and diastase resistant secretory material, coated with acid mucus which contained both sulphate and carbonyl groups. The amount of mucus diminished with increasing malignancy as did the ratio of neutral to acid mucus. Mucin histochemistry in tumours with and without argyrophil cells in a given group of malignancy was similar, and the ages of the patients having these tumours did not differ significantly.

Key words: Argyrophil and argentaffin cells, mucin histochemistry, ovarian mucinous cystadenoma.

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Received 10.11.78 Accepted 3.1.78

About 19 per cent of all ovarian tumours (Kerr & McKen 1960) are mucinous cystadenomas containing columnar epithelial cells filled with mucus as a prominent component. They belong to the «common» epithelial tumours of the ovary, and are classified morphologically into benign tumours, tumours of borderline malignancy and malignant tumours according to cellular atypia, proliferative activity and invasive capacity of the epithelial cells (Jern et al. 1973). Mucin production seems to diminish with increasing malignancy (Long & Sommers 1968). Argyrophil and argentaffin cells (here called collectively AR-cells) as well as goblet and occasional Paneth cells have been found in mucinous ovarian tumours (Morrison 1938; Fox et al. 1964 and Scull 1970). Mucinous tumours in which the epithelium contains AR-cells resembling the epithelium of the intestinal canal are classified as «enteric» tumours, and those without AR-cells as «non-enteric» tumours. The former were considered teratomatous in origin and the latter metaplastic

and colonic by Fox et al. (1964). Since the teratomatous tumours should be present already at birth it has been assumed that patients having these tumours were younger than patients having metaplastic tumours (Novak & Woodruff 1974). In another study the epithelium without AR-cells resembled that of the endocervical canal. These tumours and the tumours with AR-cells were regarded as metaplastic in origin (Fenoglio et al. 1975).

The present investigation was undertaken in order to study whether the tumours with AR-cells differ in their main histochemical characteristics from those without AR-cells, and whether there are differences in the ages of patients with different types of mucinous ovarian tumours. In order to define further criteria for the histopathological differential diagnosis between various ovarian mucinous tumours two additional aspects were investigated, namely the correlation of the grade of malignancy to mucin production on one hand and to the presence of AR-cells on the other.

of hyperploid tumour cells paralleled a good effect of hormone therapy (Kjaer *et al* 1977).

This may indicate that also patients in clinical stage I-II with hyperploid tumour cells could benefit from early treatment.

The authors wish to thank Mrs Bente Kierkegaard and Mrs Lise Wagner for excellent technical and secretarial assistance.

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PATHOLOGY OF MUCINOUS OVARIAN CYSTADENOMAS

1. Argyrophil and Argentaffin Cells and Epithelial Mucosubstances

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The presence of argyrophil and argentaffin cells and epithelial mucosubstances was studied in 187 mucinous ovarian cystadenomas of varying malignancy. Sixty-two per cent of the benign tumours contained these cells. The corresponding figures for tumours of borderline malignancy and malignant tumours were 60 and 51, respectively. The epithelial cells of the mucinous cystadenomas characteristically contained periodic acid-Schiff reactive and diastase resistant secretory material, mixed with acid mucins which contained both sulphate and carboxyl groups. The amount of mucin diminished with increasing malignancy as did the ratio of neutral to acid mucins. Mucin histochemistry in tumours with and without argyrophil cells in a given group of malignancy was similar and the ages of the patients having these tumours did not differ significantly.

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About 19 per cent of all ovarian tumours (Kerr & McKen 1960) are mucinous cystadenomas containing columnar epithelial cells filled with mucin as a prominent component. They belong to the mucous-secreting epithelial tumours of the ovary and are classified morphologically into benign tumours, tumours of borderline malignancy and malignant tumours according to cellular atypia, proliferative activity and invasive capacity of the epithelial cells (Serov *et al.* 1973). Mucin production seems to diminish with increasing malignancy (Loug & Sammers 1968). Argyrophil and argentaffin cells (here called collectively AR-cells) as well as goblet and occasional Paneth cells have been found in mucinous ovarian tumours (Masson 1938, Fox *et al.* 1964 and Smith 1970). Mucinous tumours in which the epithelium contains AR-cells resembling the epithelium of the intestinal canal are classified as 'enteric tumours' and those without AR-cells as 'non-enteric tumours'. The former were considered teratomatous in origin and the latter metaplastic

and colonic by Fox *et al.* (1964). Since the teratomatous tumours should be present already at birth it has been assumed that patients having these tumours were younger than patients having metaplastic tumours (Nevak & Woodruff 1974). In another study the epithelium without AR-cells resembled that of the endocervical canal. These tumours and the tumours with AR-cells were regarded as metaplastic in origin (Uenaglo *et al.* 1975).

The present investigation was undertaken in order to study whether the tumours with AR-cells differ in their main histochemical characteristics from those without AR-cells, and whether there are differences in the ages of patients with different types of mucinous ovarian tumours. In order to define further criteria for the histopathological differential diagnosis between various ovarian mucinous tumours two additional aspects were investigated, namely the correlation of the grade of malignancy to mucin production on one hand and to the presence of AR-cells on the other.

MATERIAL AND METHODS

All mucinous cystadenomas in the files of the Department of Pathological Anatomy University of Turku Turku Finland from 1946 to 1976 were reviewed and classified according to the WHO classification (Serov *et al* 1973). If a tumour had two or more of the morphological features mentioned below and if there was no obvious invasion, it was classified as a tumour of borderline malignancy. These features were stratification of the epithelial cells, clustering, mitotic activity and nuclear abnormalities of the cells. If invasion was observed the tumour was classified as malignant. All other tumours were classified as benign. The total material consisted of 452 cases. Of these tumours 187 were stained for the characterization of the epithelial mucosubstances and detection of argyrophil, argentaffin and Paneth cells.

The specimens were fixed in formalin for at least 24 hours, embedded in paraffin and sectioned at 5 μ m. Consecutive sections were stained with van Gieson Haematoxylin for general studies and with specific histochemical methods in order to identify the cells and the secretory products. Argyrophil cells were detected with the Bodian protargol and Grimelius silver methods (Grimelius 1968) and argentaffin cells with the Masson-Fontana method. Paneth cells were studied by the Bens carnine method after methylation for 12 hours (Lauren & Sorvari 1967). Δ -glycols (1,2-hydroxyl groups) were stained with the periodic acid-Schiff reaction (PAS). Parallel sections were treated with the Schiff reagent without periodic oxidation to exclude pre-existing aldehyde groups. The Alcian Blue, pH 2.5 PAS, after diastase digestion (AB pH 2.5-dPAS) method was used for detecting acid (blue) and neutral (red) mucosubstances. The High-iron diamine-AB pH 2.5 (HID-AB pH 2.5) method distinguishes sulphomucin (black or brown) from carbonyl mucin (blue) (Spicer 1965). Sulphomucin is also stained with AB at pH 1.0 (Lien & Spicer 1964). Salivase digestion (Neutrumsalivase, Behringwerke AG) followed by HID-AB pH 5 was carried out for detecting sialic acid residues, and hyaluronidase digestion (Hyaluronidase, Sigma) followed by AB pH 2.5 was carried out for detecting hyaluronic acid and chondroitin sulphate A and C (Luna 1968). Glycogen was demonstrated by PAS staining after digestion with a 1 per cent

aqueous solution of malt diastase (Diastase, Merck) dPAS.

The average staining intensity and the distribution of mucin in the lumen, apical border and apical, middle and basal thirds of the cell cytoplasm was estimated visually in the sections. AR-cells were considered to be present if one or more AR-cells could be identified with certainty. The results were analyzed statistically by using the Student's *t*-test or analysis of variance.

RESULTS

A Age Distribution

The present material of 452 mucinous ovarian tumours consisted of 352 benign tumours, 57 tumours of borderline malignancy and of 43 malignant tumours. In the last group there were 70 well 15 moderately and 8 poorly differentiated tumours. The patients having malignant tumours were older than the patients having benign or borderline tumours. The difference between the mean ages of the patients having benign and malignant tumours was statistically highly significant ($p < 0.001$) (Table 1).

B Argyrophil and Argentaffin Cells

Triangular cubic or elongated AR-cells were located on the basal membrane between the neoplastic epithelial cells (Fig. 1). The AR-cells did not proliferate. The tumours containing argentaffin cells also contained argyrophil cells. The results are presented in Tables 2 and 3. There were no significant differences in the mean ages of the patients having mucinous cystadenomas with and without AR-cells.

C Epithelial Mucosubstances in Benign Mucinous Cystadenomas

Of the 41 benign mucinous cystadenomas examined with various staining methods, 70 contained AR-cells whereas 21 did not. The staining results in both groups were very similar.

TABLE 1 The Age Distribution of the Patients Having Mucinous Ovarian Cystadenomas (Mean \pm S.D.)

Tumour type	Number of cases	Age (mean \pm S.D.)	Per cent
Benign tumours	352	43.7 \pm 15.5	77
Tumours of borderline malignancy	57	47.8 \pm 15.5	13
Malignant tumours	43	53.1 \pm 13.6	10
Total	452		100

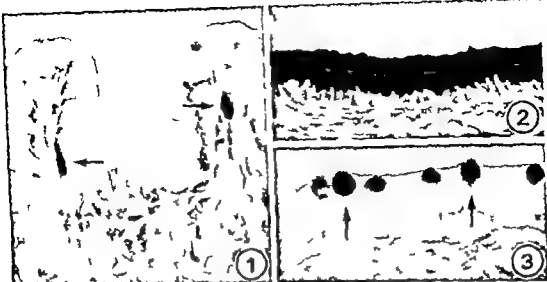


Fig. 1 Argyrophil cells (arrow) on the basal membrane between the columnar epithelial cells in a benign mucinous cystadenoma of the ovary (Bodian-protargol $\times 410$)

Fig. 2 Mucin filled regular epithelial cells in a benign mucinous cystadenoma (HPAS $\times 400$)

Fig. 3 Goblet cells (arrow) in a benign mucinous cystadenoma. The darker shade in this black and white micrograph indicates sulphomucin and the lighter shade carboxymucin (HID-AB pH 2.5 $\times 410$)

and are presented in Table 4. The epithelium in both groups was composed of columnar epithelial cells and some goblet cells. The columnar epithelial cells were rich in finely granular material containing visciglycans (Fig. 2) and some acid compounds, in which sulphomucin and carboxymucin were present in similar amounts. No Schiff reactive aldehyde groups were present without periodic acid oxidation. There were no cells containing acid mucin only. The apical border of the cells contained more acid than neutral mucin and sulphomucin domi-

nated in the former. Goblet cells contained more acid mucin than the epithelial cells (Fig. 3). Sulphate groups were present in abundance. Slalidase digestion was performed in specimens with large amounts of carboxymucin and most of it was removed by slalidase treatment. Glycogen, hyaluronic acid and chondroitin sulphate A or C were not present in the benign tumours. No Paneth cells were found. The confluent secretory material in the lumina stained similarly as the mucin within the cells.

TABLE 2. *Argyrophil and Argentaffin Cells in the Mucinous Ovarian Cystadenomas*

Tumour type	Number of cases	Argyrophil cells cases per cent		Argentaffin cells cases per cent	
Benign tumours	103	64	62	28	27
Tumours of borderline malignancy	45	27	60	22	50
Malignant tumours	39	20	51	15	38
Total	187	111	58	65	38

TABLE 3 *Number and Age Distribution of the Patients Having Mucinous Ovarian Cystadenomas with and without Argyrophil and Argentaffin Cells (Mean \pm S.D.)*

Tumour type	Argyrophil cells	number of cases	Age (mean \pm S.D.)	Argentaffin cells	Number of cases	Age (mean \pm S.D.)
Benign tumours	present	64	46.0 \pm 16.3	present	28	47.6 \pm 16.6
	absent	39	42.4 \pm 13.1	absent	75	43.3 \pm 14.5
Tumours of borderline malignancy	present	27	45.5 \pm 15.8	present	22	46.6 \pm 15.7
	absent	18	51.1 \pm 15.1	absent	23	48.8 \pm 15.8
Malignant tumours	present	20	55.3 \pm 14.0	present	15	56.6 \pm 14.1
	absent	19	52.4 \pm 12.6	absent	24	52.2 \pm 12.6
Total		187			187	

D *Epithelial Mucosubstances in Tumours of Borderline Malignancy*

The staining results of the 17 tumours with AR cells, and the 7 tumours without them were similar (Table 4). Mucin production was less abundant than in the benign tumours and was confined to the apical and middle third of the cytoplasm (Fig. 4). Acid mucin was present in larger amounts than in the benign tumours. Otherwise the staining results were similar to those in the benign mucinous cystadenomas. Some borderline tumours as well as some malignant tumours (Fig. 6) contained intrac-

epithelial vacuoles filled with mucin which stained similarly as that in lumina and with in the cells.

E. *Epithelial Mucosubstances in Malignant Mucinous Tumours*

Seventeen of the 30 malignant tumours contained AR-cells, whereas 13 did not. The histochemical staining method for mucin gave similar results both in tumours with and without AR-cells (Table 4). Mucin was observed in the apical cell border or in the apical third of the cytoplasm (Fig. 5). Mucin was totally absent from the proliferating or anaplastic

TABLE 4 *Intensity of the Histochemical Staining Reactions of the Epithelial Cells in the Mucinous Ovarian Cystadenomas*

	Benign tumours		Tumours of borderline malignancy		Malignant tumours	
	Argyrophil and Argentaffin cells					
	present	absent	present	absent	present	absent
Number of cases	20	21	17	7	17	13
Neutral mucin	+++	+++	++	++	+	+
Acid radicals	+	+	+	+	+	+
- COO ⁻	+	+	+	+	+	+
- O-SO ₂	+	+	+	+	+	+
Sialic acid	+—	+—	+—	+—	+—	+—
Glycogen	—	—	—	—	—	—
Paneth cells	—	—	—	—	—	—

Symbols - no staining or no cells, +- trace, + weak, ++ moderate and +++ strong staining. The results are averages of the stainings in each specimen as estimated visually. The decrease of neutral mucin with increasing malignancy of the tumours is statistically highly significant ($p < 0.001$).

TABLE 3 *Number and Age Distribution of the Patients Having Mucinous
Argyrophil and Argentaffin Cells (Mean \pm S.D.)*

Tumour type	Argyrophil cells	number of cases	Age (mean \pm S.D.)	Argentaffin cell
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Acid radicals	+	+	+	+	+	+
- COO ⁻	+	+	+	+	+	+
- O-SO ₃ ⁻	+-	+-	+-	+-	+-	+-
Sialic acid	-	-	-	-	+-	+-
Glycogen	-	-	-	-	-	-
Paneth cells	-	-	-	-	-	-

Symbols: - no staining or no cells, +- trace, + weak, ++ moderate and +++ strong staining. The results are averages of the stainings in each specimen as estimated visually. The decrease of neutral mucin with increasing malignancy of the tumours is statistically highly significant ($p < 0.001$).



Fig 4 Mucin in the apical part of the cells in a mucinous cystadenoma of borderline malignancy. Goblet cells (arrows) contain dPAS-positive mucin (dPAS $\times 410$)

Fig 5 A malignant mucinous cystadenoma of the ovary. Mucin is abundant only in the lumen and only sporadically found in the cells. Some goblet cells are also present (arrows) (dPAS $\times 410$)

cells. When the transitional zone, where the benign epithelium turned into a malignant one, was studied in the same tumour it was observed that there was less mucin in the malignant epithelium. The acid mucins consisted of both sulpho- and carboxy mucins with the latter in slight excess. Most of the carboxymucins contained sialic acid. The mucin in the lumina, intraepithelial vacuoles (Fig 6) and goblet cells was more acid than that in the benign and borderline tumours. The apical border of the cells contained equal amounts of sulpho- and



Fig 6 Cysts of various sizes in a malignant mucinous ovarian cystadenoma. The mucin in the cysts and on the apical cell border stains mostly black, indicating the presence of more sulphate than carboxyl groups (HID-AB pH 2.5 $\times 410$)

carboxymucins. Only traces of glycogen were found and no Paneth cells could be identified. Hyaluronidase digestion did not change the staining reactions in the epithelial cells.

The decreased amount of neutral mucin with increasing malignancy of the tumours was statistically significant ($p < 0.001$).

DISCUSSION

The majority (76.9 per cent) of the studied mucinous tumours were benign cystadenomas which is in agreement with earlier studies (Kent & McKay 1960 and Lauren et al. 1968). The proportion of borderline and malignant tumours was also similar to that reported earlier (Lauren et al. 1968 and Hart & Norris 1973).

The distribution of argyrophil cells in the benign tumours correlate well with the previously reported findings (Fox et al. 1964), but there are no figures in the literature for borderline and malignant tumours. It has been proposed that patients having mucinous cystadenomas with AR-cells are younger than patients having tumours without AR-cells, indicating that the former tumours would be of teratomatous origin (Novak & Woodruff 1974). However the present study could not confirm such an age distribution.

In a given group of malignancy the staining properties of the mucin in the epithelial cells and in the lumina were similar whether or not the tumours

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- O-SO ₃ ⁻	+-	+-	+-	+-	+-	+-
Sialic acid	-	-	-	-	+-	+-
Glycogen	-	-	-	-	-	-
Paneth cells	-	-	-	-	-	-

Symbols: - no staining or no cells, +- trace, + weak, ++ moderate and +++ strong staining. The results are averages of the stainings in each specimen as estimated visually. The decrease of neutral mucin with increasing malignancy of the tumours is statistically highly significant ($p < 0.001$).



Fig 4 Mucin in the apical part of the cells in a serous cystadenoma of borderline malignancy. Goblet cells (arrows) contain dPAS-positive mucin (dPAS $\times 410$).

Fig 5 A malignant mucinous cystadenoma of the ovary. Mucin is abundant only in the lumen and only sporadically found in the cells. Some goblet cells are also present (arrows) (dPAS $\times 410$).

cells. When the transitional zone, where the benign epithelium turned into a malignant one, was studied in the same tumour it was observed that there was less mucin in the malignant epithelium. The acid mucin consisted of both sulpho- and carboxymucins with the former in slight excess. Most of the carboxymucins contained sialic acid. The mucin in the lumen, intracanalicular acroles (Fig 6) and goblet cells was more acid than that in the benign and borderline tumours. The apical border of the cells contained equal amounts of sulpho- and



Fig 6 Cysts of various sizes in a malignant mucinous ovarian cystadenoma. The mucin in the cysts and on the apical cell border stains mostly black, indicating the presence of more sulphate than carboxyl groups (HID-AB pH 2.5 $\times 410$).

carboxymucin. Only traces of glycogen were found and no Paneth cells could be identified. Hyaluronidase digestion did not change the staining reactions in the epithelial cells.

The decreased amount of neutral mucin with increasing malignancy of the tumours was statistically significant ($p < 0.001$).

DISCUSSION

The majority (76.9 per cent) of the studied mucinous tumours were benign cystadenomas which is in agreement with earlier studies (Kent & McKay 1960 and Lauren et al 1968). The proportion of borderline and malignant tumours was also similar to that reported earlier (Lauren et al 1968 and Hart & Norris 1973).

The distribution of argyrophil cells in the benign tumours correlate well with the previously reported findings (Fox et al 1964), but there are no figures in the literature for borderline and malignant tumours. It has been proposed that patients having mucinous cystadenomas with AR-cells are younger than patients having tumours without AR-cells, indicating that the former tumours would be of teratomatous origin (Novak & Woodruff 1974). However the present study could not confirm such an age distribution.

In a given group of malignancy the staining properties of the mucin in the epithelial cells and in the lumens were similar whether or not the tumours

contained AR-cells. The amount of mucin decreased with increasing malignancy of the tumours as observed earlier by Long & Sommers (1968). In addition the ratio of neutral to acid mucins decreased with increasing malignancy and this may aid in the differential diagnosis of these tumours. However there was no distinct line between benign and borderline tumours or between the latter and malignant ones. The staining reactions of goblet cells were similar in tumours with and without AR cells.

From the present observations it can be concluded that in a given group of malignancy the ages of the patients having mucinous ovarian tumours with and without argyrophil cells and the staining properties of the mucin in the tumours with and without argyrophil cells are similar. Thus all mucinous tumours in this study actually seem to belong to the same group. The histogenesis of the mucinous ovarian tumours will be discussed in the accompanying paper dealing with the ultrastructure of these tumours (Alemi & Aivalainen 1978).

This work was supported by a grant from the Emil Aaltonen Foundation. The author is grateful to Mrs. Anja Tuoristo and Mr. Yrjö Lehtimäki for skilled technical assistance.

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PATHOLOGY OF MUCINOUS OVARIAN CYSTADENOMAS

2 Ultrastructural Findings

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Klemi, P. J. & Nevalainen, T. J. Pathology of mucinous ovarian cystadenomas. I. Ultrastructural findings. Acta path. microbial. scand. Sect. A, 86: 471-481, 1978.

Fourteen mucinous ovarian cystadenomas of different grades of malignancy (7 benign, 4 borderline and 3 malignant) were investigated by electron microscopy. The main tumour cell type was a columnar mucous epithelial cell with short microvilli. With increasing grade of malignancy the shape and size of these cells became more irregular and the number of mucous granules in them decreased. Most of the mucous granules had a dense core and a less dense reticular component, which stained well with the periodic acid-silver methenamine (PAS-1) technique. Goblet cells with the usual ultrastructure were found rather frequently. The tumours in a given group of malignancy were relative similar to each other in their light and electron microscopic appearance. However a number of tumours contained argyrophil and argentaflin cells in addition to the columnar and goblet cells. The findings of the present study support the view that the mucinous ovarian cystadenomas arise from the ovarian surface epithelium via a metaplastic process.

Key words: Argyrophil and argentaflin cells, histogenesis, mucinous ovarian cystadenoma, ultrastructure.

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Received 10 x 78 Accepted 3 78

Mucinous ovarian cystadenomas belong to the mucinous epithelial tumours of the ovary and are classified into benign tumours, tumours of border line malignancy and malignant tumours (Savoy *et al.* 1973). Histochemical characteristics of these tumours were discussed in the preceding article (Klemi 1978). There are few ultrastructural studies dealing with benign mucinous ovarian cystadenomas (Lazlo *et al.* 1977 and Fowglio *et al.* 1975), and one with their malignant counterparts (Joachim *et al.* 1975) but none with the tumours of borderline malignancy. The purpose of the present work was to describe the ultrastructure of benign, borderline and malignant mucinous cystadenomas of the ovary in an attempt to study the histogenesis of these tumours. In order to confirm the previous histochemical and ultrastructural findings of the epithelium of the endocervical canal, samples of normal endocervix were investigated.

MATERIAL AND METHODS

Multiple samples from 14 surgically removed ovarian mucinous cystadenomas (7 benign, 4 of borderline malignancy and 3 malignant ones) and 3 biopsies from normal endocervical epithelium were processed for light and electron microscopy. For light microscopy the specimens were stained with various histochemical methods to characterize the epithelial mucopolysaccharides and various cells as described in the preceding article (Klemi 1978).

For electron microscopy the tissues were fixed in 3 per cent glutaraldehyde in 0.1 cacodylate buffer at pH 7.4 for 3-96 hours and postfixed in 1 per cent osmium tetroxide in the same buffer for 1 hour. The tissues were washed in buffer, dehydrated in ethanol, and embedded in Epon 812 (Luft 1961). Semithin sections were stained with alkaline toluidine blue and PAS technique (Nevalainen *et al.* 1972) for orientation purposes and for counting of nucleoli. Ultra-thin sections of Epon embedded tissues were stained with uranyl acetate (UA) (Hanson 1958) and lead citrate (Pb) (Reynolds &

contained AR-cells. The amount of mucin decreased with increasing malignancy of the tumours as observed earlier by Long & Sommers (1968). In addition the ratio of neutral to acid mucins decreased with increasing malignancy and this may aid in the differential diagnosis of these tumours. However there was no distinct line between benign and borderline tumours or between the latter and malignant ones. The staining reactions of goblet cells were similar in tumours with and without AR-cells.

From the present observations it can be concluded that in a given group of malignancy the ages of the patients having mucinous ovarian tumours with and without argyrophil cells, and the staining properties of the mucin in the tumours with and without argyrophil cells are similar. Thus all mucinous tumours in this study actually seem to belong to the same group. The histogenesis of the mucinous ovarian tumours will be discussed in the accompanying paper dealing with the ultrastructure of these tumours (Kleini & Heralainen 1978).

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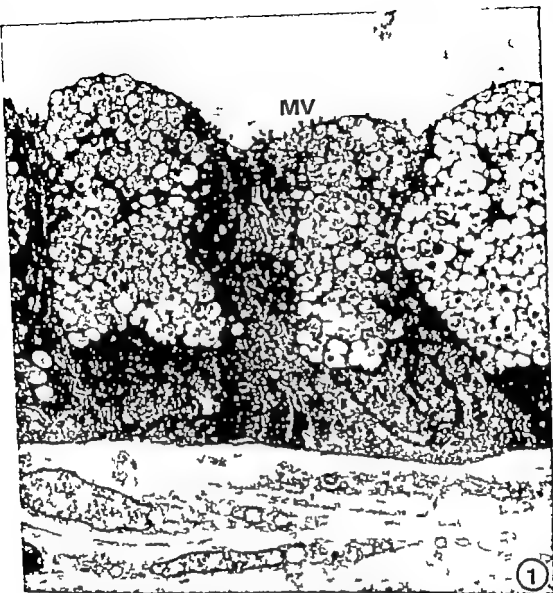


Fig. 1 An electron micrograph of columnar mucous epithelial cells in a benign mucinous ovarian cystadenoma. There are few short microvilli (MV) in the luminal cell surface. The lateral plasma membranes are convoluted. Desmosomes (D) are visible between cells. The apical cytoplasm is full of secretory granules (S) with a reticular outer part (R) and often an electron dense central core (Co). Other organelles are displaced to the periphery of the cell. The nucleus (N) is located basally. Stained with uranyl acetate (UA) and lead citrate (Pb). Mag. 5 800 \times .

granules did not stain with silver methenamine without periodic acid oxidation. In some columnar cells there were slightly larger mucous granules, up to 1.2 μ m in diameter which contained one or several electron dense cores. Elongated mitochondria and some profiles of granular and agranular endoplasmic were located in the cell periphery and infranuclear cytoplasm. A prominent Golgi complex with mucous granules of various sizes was often

visible in the lateral cytoplasm. The nucleus, located in the basal portion of the cell, was irregular in shape with deep invaginations in the nuclear membrane. The chromatin was condensed in the periphery against the nuclear membrane. (Fig. 1). Mostly the nuclei contained one nucleolus, but 15 per cent of the nuclei had two nucleoli and one per cent had three nucleoli (Table 2).

The goblet cell at varying stages of maturation

Coggeshall 1965) and studied with a JEOL JEM 100 C electron microscope. Silver methenamine staining after periodic acid oxidation (PASM staining) was performed on ultra-thin sections in order to demonstrate mucosubstances with 1,2 hydroxyl groups (vic-glycols) at the ultrastructural level (Aivalainen & Kleem 1976).

RESULTS

The histological and gynecological features of the present cases are summarized in Table 1. The ratio of neutral to acid mucin in the epithelial cells of the tumours diminished with increasing malignancy similarly to that described in the preceding article (Alemi 1978).

ELECTRON MICROSCOPICAL FINDINGS

A. Benign Mucinous Cystadenomas

The columnar mucous epithelial cell which contained numerous secretory granules, was the main cell type in the benign mucinous cystadenomas. The cell rested on the basal lamina under which there were connective tissue cells. The lateral plasma membranes were convoluted. There were tight junctions and desmosomes between adjacent epithelial cells. The luminal cell surface was

TABLE 2. The Percentages of Nucleoli in 14 Mucinous Ovarian Cystadenomas of Different Degrees of Malignancy

Type of tumour	No of cases	No of nuclei counted	Percentages of nuclei containing 1, 2, 3 or more nucleoli			
			1	2	3	more than 3
Benign	7	2000	15	1		0
Borderline	4	1000	80	17	3	0
Malignant	3	1000	78	19	2	1

relatively smooth with a few poorly developed irregular microvilli, some of which had distinct rootlets (Figs. 1 and 3).

The cytoplasm was full of mucous granules surrounded by a unit membrane. The granules were up to 0.8 μ m in diameter and often contained a core which stained densely with UA + Pb (Fig. 1). The major part of the granule was reticular material. The core did not stain with the PASM method, whereas the rest of the granule stained intensely indicating the presence of vic-glycols (Fig. 2). The

TABLE 1. Histological and Gynecological Features of Studied Cases

Case No	Age	Type of tumour	Cell types identified at the light microscopical level				Other lesions in the genital tract
			Columnar	Goblet	Argyrophil	Argentaffin	
74-6	42	Benign	+	+	+	-	-
74-7	42	Benign	+	+	-	-	-
74-13	80	Benign	+	+	-	-	Endometrial atrophy
75-28	19	Benign	+	+	+	-	-
75-29	30	Benign	+	+	+	-	-
75-36	32	Benign	+	+	-	-	Pregnant
76-3	37	Benign	+	+	-	-	-
74-14	62	Borderline	+	+	-	-	Cystic glandular hyperplasia of the endometrium, benign endocervical polyp
75-24	63	Borderline	+	+	+	+	Endometrial atrophy
75-35	60	Borderline	+	+	-	-	Endometrial atrophy epidermoid carcinoma in the cervical canal irradiated 8 years ago
76-15	61	Borderline	+	+	+	+	Benign Brenner tumour in the same ovary endometrial atrophy carcinoma in situ in the cervical canal
75-34	27	Malignant	+	+	-	-	-
76-10	70	Malignant	+	+	+	+	Endometrial atrophy
76-17	58	Malignant	+	-	-	-	Endometrial atrophy

Symbols + = present, - = absent

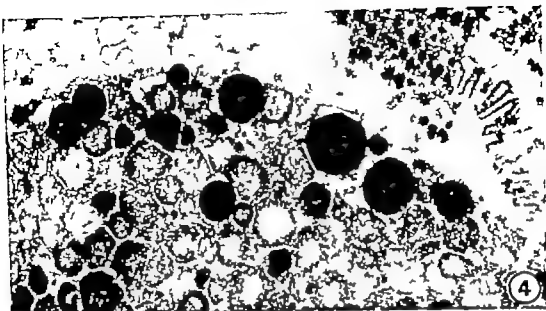


Fig 4 Part of a goblet cell in a benign mucinous cystadenoma. Most of the secretory granules stain deeply and relatively homogeneously with PAS-UV method. In some granules the staining is more intense at the periphery. Mag 16 000 \times

was another common cell type in the benign mucinous cystadenoma. The mature goblet cell was typically ovoid in shape. The free cell surface was distended bulging into the lumen of the cystadenoma and carried only few short microvilli. The

lateral plasma membrane was smooth with some well developed desmosomes. The cytoplasm was full of secretory granules up to 1.0 μ m in diameter (Fig 3). The mucous granules contained homogeneous or reticular material, but sometimes there was



Fig 5 A triangular argyrophil cell, which is typically located on the basal lamina in a benign mucinous cystadenoma. The secretory granules are round, homogeneous in texture, of various density and up to 0.4 μ m in diameter. UA + Pb. Mag 7 440 \times

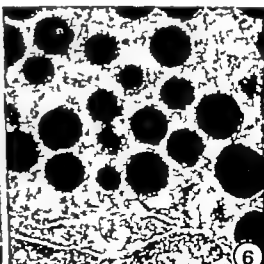


Fig 6 Higher magnification of the secretory granules in an argyrophil cell. The granules stain homogeneously and intensely and they are surrounded by a thin membrane. UA + Pb. Mag 33 450 \times

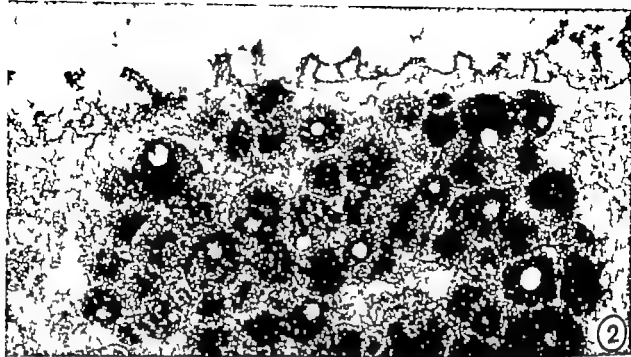


Fig 2 Secretory granules of columnar mucous cell stained with periodic acid silver methenamine method (PAS). The core does not stain, but the main part of the granule (the outer reticular part) is densely stained. Mag. 14 800 \times

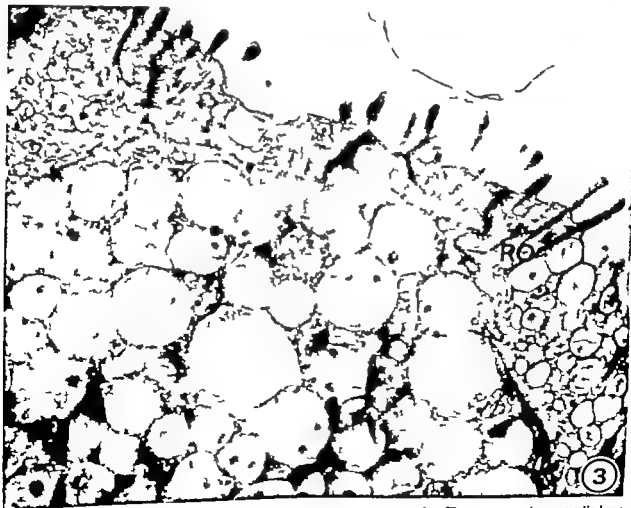


Fig 3 The apical part of a goblet cell contains homogeneous secretory granules. The adjacent columnar cells have microvilli with long rootlets (RO) in the apical cytoplasm. UA + Pb. Mag. 18 500 \times

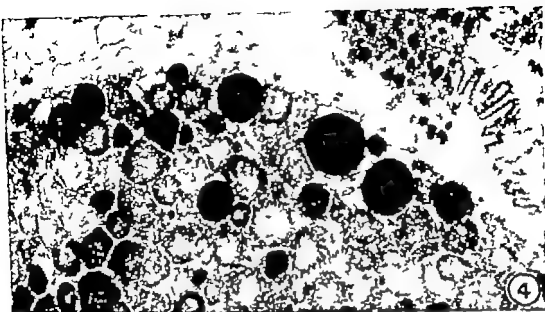


Fig 4 Part of a goblet cell in a benign mucinous cystadenoma. Most of the secretory granules stain deeply and relatively homogeneously with PASM method. In some granules the staining is more intense at the periphery. Mag. 16 000 \times .

was another common cell type in the benign mucinous cystadenoma. The mature goblet cell was typically oval in shape. The free cell surface was distended bulging into the lumen of the cystic cavity and carried only few short microvilli. The

lateral plasma membrane was smooth with some well developed desmosomes. The cytoplasm was full of secretory granules up to $1.0 \mu\text{m}$ in diameter (Fig. 3). The mucous granules contained homogeneous or reticular material, but sometimes there was

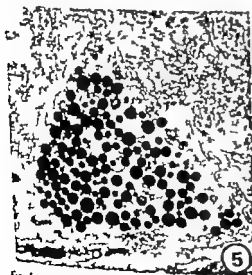


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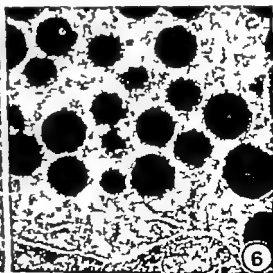


Fig 6 Higher magnification of the secretory granules in an argyrophil cell. The granules stain homogeneously and intensely and they are surrounded by a unit membrane. UA + Pb. Mag. 33 450 \times .



Fig 7 An electron micrograph of a mucinous cystadenoma of borderline malignancy. The columnar epithelial cells are more irregular in appearance than those in the benign tumours (compare to Fig 1). The nuclei (N) are irregular in shape with one or two nucleoli. Secretory granules (S) are diminished in number. UA + Pb Mag 3 950 \times

one large central core, which stained less intensely with the PASM method than did the periphery (Fig 4). Some smooth and relatively abundant rough endoplasmic reticulum, some mitochondria, free ribosomes, the Golgi complex and lysosomes were

confined to the lateral cytoplasm. The nucleus was oval or irregular in shape with chromatin condensed along the nuclear membrane.

The third cell type, the *argyrophil cell*, was triangular or cuboidal in shape and rested on the basal lamina. Argyrophil cells were found at the electron microscopical level only in one case. The plasma membrane was smooth and there were few desmosomes. The secretory granules were surrounded by a membrane, had a diameter of 0.15–0.4 μ m and were homogeneous in texture and of variable density (Figs. 5 and 6). The granules did not stain with the PASM method.

B. Cystadenomas of Borderline Malignancy

The plasma membrane of the columnar cells was folded, and tight junctions and desmosomes could be identified. The microvilli on the apical cell surface were variable in length as found also in the benign tumours. The columnar cells in the proliferating areas contained less mucous granules (Fig. 7) than corresponding cells in the benign tumours, but the granules exhibited similar ultrastructural staining characteristics. The profiles of the smooth and rough endoplasmic reticulum were rare. Glycogen was not found. Primary and secondary lysosomes were found more often than in the benign tumours. The nucleus was irregular in shape and contained mostly one nucleolus but two and three nucleoli were found more often than in the benign tumours (Table 2). In other respects the columnar and goblet cells were similar in their ultrastructure to those in the benign tumours. No argyrophil or argentaffin cells (AR-cells) were found in the electron microscope.

C. Malignant Mucinous Cystadenomas

In the malignant tumours there were cystic cavities lined with tumour cells. They had short microvilli, often with long rootlets, on the luminal cell surface, as was seen in the cells in the benign and borderline tumours. The plasma membrane was smooth and only few desmosomes could be identified. Some secretory granules were observed in the cytoplasm of the cells lining the cavities of the tumours. The granules were similar in their fine structure and staining characteristics to those found in the less malignant tumours but there were fewer of them in the malignant tumours. Mitochondria were sparse and round or oval in shape. There were numerous free ribosomes, and secondary lysosomes, most probably representing autophagic vacuoles, in the cytoplasm of the tumour cells. The Golgi complex was relatively extensive in some cells. As a



Fig 8 Two argyrophil cells (A) in malignant mucinous cystadenoma. Some epithelial tumour cells contain mucous granules (S) but most of the tumour cells lack any secretory material. The nuclei are irregular in shape and the cytoplasm is scarce. L.A. Ph Mag 3 630

rule, few profiles of smooth and rough endoplasmic reticulum were observed, but one tumour contained many cells with well developed rough endoplasmic reticulum. Little granular glycogen was found in the cytoplasm. The nuclei in the tumour cells displayed

a wide range of variation in size and shape. The nuclear membrane was often deeply indented (Fig 8). Two nucleoli per nuclei were found in 19 per cent, three in 2 per cent and more than three in one per cent of the nuclei (Table 2).



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low inner structure without electron dense cores (Fig. 9). The reticular material stained well with the PAS-lead method. The microvilli on the apical cell surface had few short rootlets.

DISCUSSION

With increasing grade of malignancy of the tumours the shape and size of the columnar epithelial cells became more irregular and the number of secretory granules diminished which agreed well with the findings at the light microscopical level (Kleml 1978). The number of nuclei increased with increasing malignancy as noticed with other tumours of the female genital tract (Long & Dole 1959 and Long & Taylor 1961). There were many desmosomes and tight junctions between the columnar cells, which is characteristic for neoplastic cells of epithelial origin, and also frequently found at the cells of the ovarian surface epithelium (Worley *et al.* 1975 and Gaudes 1975). There were no consistent ultrastructural features, which could aid the differential diagnosis between benign, borderline and malignant mucinous cystadenomas.

The microvilli on the apical cell surface resembled somewhat that of intestinal columnar cells in normal and malignant epithelia of the gastrointestinal canal (Kjær *et al.* 1973 and Verelsteden & Järn 1977). Real brush borders, typical of the intestinal absorptive cells, were not found in the cells of the mucinous ovarian cystadenomas in the present material. On the other hand typical brush borders have been reported on the apical cell surfaces in a mucinous ovarian cystadenoma with arginomatous and goblet cells (Järn 1967). Well developed long microvilli in the brush border similar to those of intestinal absorptive cells, were demonstrated in this specimen by electron microscopy (Järn & Kleml unpublished results).

The reason for the bipartite staining of the mucous granules is unknown. It may depend on the difference in the mucin (Bloom *et al.* 1977) or protein composition (Freeman 1974) of the two regions of the granule or it may be the result of uneven fixation (Miner & Reid 1970 and Tandler & Erlanson 1972). The level of sectioning may also influence the appearance of the granule in the electron microscope. However it seems less probable that the bipartite structure of the granule is caused by artefactual reasons since one can find several types of granules side by side in the same tumour. Granules with bipartite substructure were described in the mucous superficial epithelial and glandular cells of the human stomach (Ming *et al.* 1967 and Verelsteden & Järn 1977), in the mucous cells of human bronchus (Miner & Reid 1970) and

in the mucous cells of hamster submandibular gland (Bloom *et al.* 1977).

The granules in the goblet, and some columnar cells were without electron dense cores. Similar simple granules were found in the cells of the endocervical canal and have been found also in the goblet cells of small intestine (Freeman 1966) and the intestinal metaplasia in the stomach (Verelsteden & Järn 1977).

AR-cells in the mucinous cystadenomas of the present study contained secretory granules with ultrastructure typical of the cells of APUD-series, which are derived from the neural crest (Purse 1969), i.e. from another germ layer than the epithelial cells of the ovarian surface, which is mesodermal in origin.

Fox *et al.* (1964) have reviewed and discussed throughout the theories of the pathogenesis of the mucinous ovarian tumours. According to their findings and those of others (Laurier *et al.* 1972) the mucinous tumours with AR-cells are teratomatous in origin, as Merz (1938) assumed, and those without AR-cells originate from the ovarian surface epithelium and thus are metaplastic in origin. On the other hand, in a recent study (Frangulo *et al.* 1975) it has been suggested that all mucinous tumours are metaplastic in origin, some resembling the structures of the intestinal canal and some those of the endocervical canal.

The evidence for the hypothesis of the teratomatous origin of some mucinous ovarian cystadenomas is based on certain structural similarities (columnar cells with microvilli, goblet and AR cells) between the epithelium of the mucinous ovarian cystadenomas and normal intestinal epithelium, the epithelium of areas of gastric intestinal metaplasia (Ming *et al.* 1967 and Verelsteden & Järn 1977), and the intestinal metaplasia of gallbladder (Järn & Lauren 1967 and Lunde & Verelsteden 1975) and nose (Järn 1944).

If the mucinous ovarian cystadenomas with AR cells were teratomatous in origin, the epithelium of the benign tumours should be identical with that of intestinal mucosa. However this does not seem to be the case. Furthermore, the epithelium in the mucinous ovarian tumours without AR-cells and in the endocervical canal are not identical. On the other hand the findings of the present and the preceding studies (Kleml 1978) showed that the mucinous ovarian cystadenomas with and without AR-cells were similar in their histochemical and ultrastructural characteristics, and that the ages of the patients having these tumours did not differ from each other. Therefore the mucinous tumours, regardless of the presence or absence of AR-cells, form a homogeneous group and the monophyletic terato-

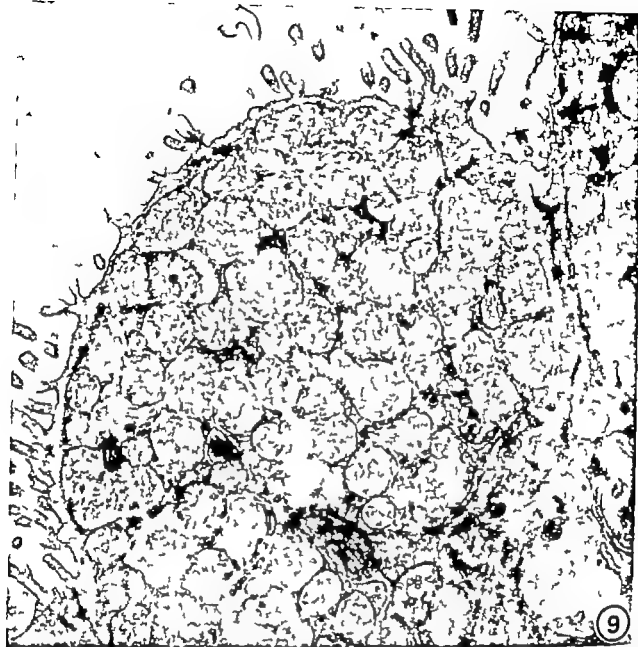


Fig 9 An electron micrograph of the apical part of a mucous columnar non-ciliated secretory cell in the epithelium of the endocervical canal. The mucous secretory granules are surrounded by a unit membrane and contain reticular material without central cores UA + Pb Mag 18 500 \times

The goblet cells displayed the same ultrastructure as was observed with the less malignant tumours.

AR-cells were round, triangular or low columnar in shape. The plasma membrane was smooth and there were only occasional desmosomes. The secretory granules were round, surrounded by a membrane, had a diameter of 0.15–0.4 μm , and varied slightly in density as did the granules in the argyrophil cells of the benign mucinous cystadenomas. There was no ultrastructural features which could aid in distinguishing argyrophil and argentaffin cells from each other.

D Epithelium of the Endocervical Canal

The epithelium of the endocervical canal was composed of ciliated and nonciliated columnar cells, and of smaller basal undifferentiated cells. The secretory material of the columnar cells contained proportionally more acid mucin with sulphate and carboxyl groups than that of the columnar cells in the benign mucinous cystadenomas. Most of the secretory material was neutral mucin. At the ultrastructural level the secretory granules in the columnar cells were up to 0.8 μm in diameter surrounded by a unit membrane, and reticular in

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matous origin of mucinous ovarian cystadenomas with AR-cells can be ruled out.

The evidence for the metaplastic origin of mucinous cystadenomas with and without AR-cells is based on the following observations. The epithelium of normal ovary does not show any mucous secretion (Gondos 1975). According to Radisavljevic (1977) the epithelium of inclusion cysts originates from the ovarian surface epithelium and its cells can transform into mucin secreting ones (Tower 1956 and von Numers 1965). Accordingly this mucinous epithelium is to be considered a result of a metaplastic process in the ovarian surface epithelium which is mesodermal in origin. It is possible that mesodermally derived epithelia, such as that in the renal pelvis, can change into intestinal type epithelia with goblet, Paneth and AR-cells (Gordon 1963). Also a normal mesodermally derived epithelium such as that in the endocervical canal may contain AR-cells (Fox *et al* 1964 and Talcshl *et al* 1975). AR-cells have also been found in two endometrioid ovarian carcinomas (Klemi & Grönlund 1978) which are mesodermal in origin (Cummins *et al* 1974). Therefore the presence or absence of AR-cells alone is not a sufficient finding for dividing the tumours histogenetically into two different groups. It can be concluded that there is only one histogenetic type of mucinous ovarian cystadenomas and that all these tumours are metaplastic in origin.

The study was supported by a grant from the Emil Aaltonen Foundation. The authors thank Miss P. Kulmala, Miss E. Saarinen and Mr M. Lehmäki for their skilful technical assistance.

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OBSERVER VARIATION IN HISTOLOGIC CLASSIFICATION OF THYROID CANCER

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Saxen, E., Fransilla, A., Bjarnason, B., Normann, T. & Ringertz, N. Observer variation in histologic classification of thyroid cancer. *Acta path. microbiol. scand. Sect. A*, 86 483-486, 1978.

Histologic slides of 696 cases of thyroid cancer reported to the national cancer registries of Finland, Iceland, Norway and Sweden were reviewed by 5 Nordic pathologists in order to determine the observer variation between the pathologists, as well as the reproducibility of the WHO classification of thyroid tumours. In 58% of the cases all observers agreed upon the diagnosis and in 82% at least three of them agreed. The observer disagreement was lowest for papillary carcinoma (7%) and highest for follicular carcinoma (27%). The corresponding figures for anaplastic and medullary carcinomas were 18% and 23% respectively. The most common diverging diagnosis for cases finally interpreted as papillary carcinoma was follicular carcinoma, and for cases finally interpreted as follicular carcinoma, a benign thyroid lesion. The results of the present study clearly indicate the necessity of having all cases reviewed by the same pathologist or group of pathologists in order to obtain reliable results for comparative studies.

Key words: Thyroid cancer, histologic classification, observer variation, quality control in histopathology.

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Received 17 xi 78 Accepted 4 iv 78

The macroscopic features of tumours currently form the basis for their classification. Despite the use of standardized nomenclatures and standard histologic criteria, it is well known among pathologists that observer variation occurs in the histologic classification of tumours and that this variation is sometimes considerable. For this reason the results of many comparative studies on incidence based on the histologic typing of tumours may be unreliable.

The purpose of the present study was to investigate the reproducibility of the WHO histologic classification of thyroid tumours (6) and to determine the extent of observer variation among 5 Nordic pathologists. The present study is the first of two reports on the incidence of different types of thyroid cancer in Finland, Iceland, Norway and Sweden.

MATERIAL AND METHODS

The study included all cases of thyroid carcinoma reported to the national cancer registries in Finland (1959-61), Iceland (1955-68), Norway (1959-60), and Sweden (1960) (Table 1), a total of 713 histologically diagnosed cases. During these years between 93 and 96 per cent of the thyroid cancers registered were confirmed histologically. Histologic slides were available for 696 cases.

For the sake of simplicity only one or a few representative slides were chosen from each case. The hematoxylin-eosin and Wiegert van Gieson staining methods were used. The tumours were placed into the following groups according to the WHO histological classification of thyroid tumours (6): 1. follicular, 2. papillary, 3. anaplastic, 4. medullary, 5. non-specified carcinoma, 6. lymphoma or sarcoma, 7. not thyroid cancer (atypical adenomas, benign lesions and metastatic tumours), and 8. no diagnosis possible.

TABLE 3 Degree of Unanimity between the Participating Pathologists, by Final Histologic Type

Final diagnosis	No. of cases	Degree of unanimity				
		4	3+1	2+2	2+1+1	1+1+1+1
Follicular carcinoma	111	26.1	43.2	15.3	13.5	1.8
Papillary carcinoma	327	81.0	10.4	4.9	2.8	0.9
Anaplastic carcinoma	123	30.4	30.1	8.1	7.3	4.1
Medullary carcinoma	31	45.2	22.6	9.7	19.4	3.2
Sarcoma & lymphoma	7	14.3	57.1	28.6	—	—
Not thyroid cancer	87	28.7	39.1	13.8	12.6	5.7
All cases	696	58.3	23.9	8.5	7.3	2.3

4 = all pathologists of the same opinion

1+1+1 = all pathologists of different opinions

carcinoma was, however, its separation from benign thyroid lesions and atypical adenomas.

The proportion of observer disagreement was 18% for anaplastic carcinoma. The diverging diagnoses were distributed rather evenly among the other histologic types of cancer. All four pathologists were of the same opinion in only 50% of such cases. The disagreement was even more pronounced for medullary carcinoma. Diverging diagnoses accounted for 23% with all pathologists of the same opinion in only 45% of the cases. The most common diverging diagnosis in that group was anaplastic carcinoma.

The diverging diagnoses in cases with a final diagnosis of malignant lymphoma was anaplastic carcinoma in all cases.

Eighty-seven cases, i.e. 12.5% of the whole series, received the final diagnosis "not thyroid cancer." Sixty-six of these were interpreted as atypical adenomas. The proportion of diverging diagnoses in this group was 27%. The most common diverging diagnosis was follicular carcinoma.

DISCUSSION

Different histologic types of tumour of an organ may be an indication of differences in epidemiology, aetiology and clinical features. A great deal of information is therefore lost if malignant tumours of one specific site are treated as a single homogeneous group in epidemiologic and clinical studies. Differences between studies dealing with tumours of a specific site may be explained by the varying frequencies of different histologic types in the series. For these reasons it seems inappropriate to draw far-reaching conclusions from comparative studies if the tumours are not classified histologi-

cally. The observer variation in the histologic classification of tumours may however be so great that it seems relevant to raise serious doubts about the validity of many clinical trials and epidemiologic investigations into the incidence of different histologic types of tumours. A high level of observer disagreement has been shown in studies dealing with lung cancer (3, 10), cervical cancer (11), malignant lymphoma (2, 5, 7, 9) and thyroid tumours (4).

The results of the present study illustrate some of the difficulties in the classification of thyroid tumours. Although the participating pathologists were well trained in thyroid pathology and used the same standard histologic nomenclature and classification, total agreement in the histologic classification was achieved in only 58% of cases. In particular, the study illustrated the well-known fact that it is difficult to judge malignancy in follicular tumours. Only in one fourth of the cases where the final diagnosis was follicular carcinoma were all the participating pathologists of the same opinion, the most common diverging diagnosis was a benign thyroid lesion. On the other hand, the problem is also illustrated by the fact that follicular carcinoma was the most common diverging diagnosis in cases where the final diagnosis was a benign lesion.

The study illustrates the diagnostic problem in medullary carcinoma described earlier (8). In the present study it proved especially difficult to differentiate medullary carcinoma from anaplastic carcinoma (no special stains were used).

The diagnosis of papillary carcinoma seemed to be the one most easily agreed upon. The rare diverging diagnosis was almost always follicular carcinoma. In most cases, however, it was possible to differentiate between follicular carcinoma and papillary carcinoma with almost exclusively follicu-

TABLE 1 *Number of Histologically Diagnosed Cases of Thyroid Cancer Reported to the National Cancer Registries of Four Nordic Countries*

Years	No. of cases		
	Males	Females	Total
Finland 1959-1961	43	157	200
Iceland 1955-1968	29	110	139
Norway 1959-1960	56	98	154
Sweden 1960	61	159	220
Total	189	524	713

All cases from every country were reviewed by one experienced pathologist from each country. The pathologists had no knowledge of clinical features, the original histologic diagnoses or the diagnoses made by the other participating pathologists.

When three or all four pathologists made the same diagnosis, this was accepted as the final diagnosis. The remaining cases were discussed at a joint meeting and the correct final diagnosis was determined for each case. There were thus four individual diagnoses and one final diagnosis for each case, for a total of 7784 individual and 696 final diagnoses.

RESULTS

Table 2 shows the percentage distribution of all 2784 diagnoses given, by final histologic type, and Fig 1 the percentage of diverging diagnoses in the different histologic types. Diverging diagnoses (diagnoses that differed from the final diagnosis)

accounted for 14% of the total series. In 58% of cases all observers agreed, and in 82% at least three of them were in agreement (Table 3).

The percentage of observer disagreement was lowest for papillary carcinoma (7%) and highest for follicular carcinoma (27%). In 81% of cases where the final diagnosis was papillary carcinoma, all four pathologists agreed upon the diagnosis in contrast to only 26% in the group where the final diagnosis was follicular carcinoma (Table 3).

In cases where the final diagnosis was papillary carcinoma the diverging diagnosis was follicular carcinoma in the majority of cases, while the diverging diagnoses of cases finally interpreted as follicular carcinoma were evenly distributed into papillary, anaplastic and medullary carcinoma. The most common problem in the diagnosis of follicular

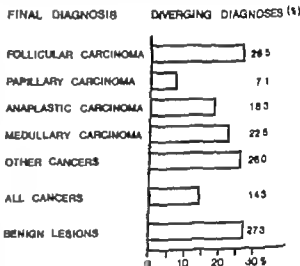


Fig 1 Percentage of diverging diagnoses, by final histologic type.

The two Finnish pathologists gave a joint diagnosis

TABLE 2 *Percentage Distribution of the 2784 (4 x 696) Diagnoses Given by the Participating Pathologists, by Final Histologic Type*

Final diagnoses		No. of cases	Diagnoses given							No Dg
			F	P	A	M	C	S&L	N	
Follicular carcinoma	(F)	111	73	6	2	5	0	-	11	1
Papillary carcinoma	(P)	327	4	93	0	0	0	-	1	1
Anaplastic carcinoma	(A)	123	4	3	82	1	3	2	3	1
Medullary carcinoma	(M)	31	3	2	10	77	8	-	-	2
Carcinoma NOS	(C)	10	5	-	18	3	55	-	18	3
Sarcoma & Lymphoma	(S&L)	7	-	-	29	-	-	71	-	-
Not thyroid cancer	(N)	87	14	4	2	2	1	-	73	4
Total		696								

CYTOPLASMIC EFFECTS OF X IRRADIATION ON CULTURED CELLS IN A NONDIVIDING STAGE

3 Alterations in Plasma Membrane Motility

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Hamberg, H., Brunk, U., Ericsson, J. L. E. & Jung, B. Cytoplasmic effects of X-irradiation on cultured cells in a nondividing stage. 3 Alterations in plasma membrane motility. *Acta path. microbiol. scand. Sect. A*, 86: 487-494 1978.

Cultured, density-dependent growth inhibited human glioma cells were exposed to X radiation, generated by an 8-MeV linear accelerator at a dose of 200 Gy. Phase contrast microscopy, time-lapse cinematography and scanning electron microscopy showed the irradiated cells to have increased ruffling activity of plasma membranes and enhanced macropinocytosis with a maximum approximately 24 hours after irradiation. Atypical central ruffles arising from the upper cell surface were demonstrated on some irradiated cells. The turnover of plasma membranes was supposed to be increased in the irradiated cells resulting in the formation of the observed branched, thread-like cells. The detached cell surface area was believed to result from an imbalance between degradation and renewal of the plasma membrane in the irradiated cells.

Key words: X-irradiation, cultured human cells, plasma membrane motility, scanning electron microscopy, time-lapse filming.

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Accepted as submitted 16.7.78

Cultured human glioma cells of astrocytic origin, blocked in G₁ by density inhibition, are relatively dose-resistant and the cells survive a rather extensive radiation dose for many days. Only following a dose of about 200 Gy do unequivocal cytoplasmic alterations occur within one or two days in a degree making the study of early changes comparatively easy with transmission electron microscopy (TEM) (Hamberg *et al.* 1976). Cells exposed to such radiation damage showed enhanced autophagocytosis and endocytosis associated with the formation of large intracytoplasmic vacuoles in the vicinity of the nucleus. The alterations were interpreted as signs of degradative and reparative processes resulting in increased turnover of various cytoplasmic organelles including the plasma membrane (Hamberg *et al.* 1977).

In non-irradiated glioma cells, extensive endocytosis occurs mainly during the logarithmic growth phase and then often in portions of the cells showing ruffling activity (Brunk *et al.* 1976, Schellens *et al.* 1976). When glioma cells are blocked in G₁, either due to serum starvation in sparse cultures or density dependent growth inhibition following confluency

ruffling and associated endocytotic activity are abolished (Lofgren *et al.* 1977). The irradiated cells seemed to enhance their endocytotic activity as judged from TEM studies of confluent monolayers (Hamberg *et al.* 1977). We therefore deemed it necessary to analyse irradiated cells with methods that permit the study of cell surface motility and configuration more readily and completely than what is possible with TEM. The aim of the present study was to follow early reparative processes caused by heavy X-irradiation with the combined use of scanning electron microscopy (SEM) and time-lapse cinematography.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

All experiments were performed on cells cultivated diploid, human normal glioma cells of the U 787 CG line, using only cells from early passages in phase II. The cell line was established in culture by B. Westermarck, The Wallenberg Laboratory, Uppsala, as described by Powers and Macintosh (1962). Cells were grown in Eagle's minimal essential medium supplemented with 10% calf

lar growth pattern by the recognition of «ground class nuclei» at least in some areas, in the latter

The results of the present study clearly indicate the necessity of having all cases reviewed by the same pathologist or group of pathologists in order to obtain comparable data.

The Project was supported by funds provided in part by the International Cancer Research Data Bank Programme of the National Cancer Institute National Institutes of Health (US), under Contract No NO1 CO-65341 with the International Union Against Cancer

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serum and antibiotics, and maintained in humidified air containing 5% CO₂ at 37° C. The cells were grown in plastic Petri dishes, some of which contained round 25 mm glass coverslips No. 0 (for time-lapse cinemicrography) or 12 × 6 × 1 mm glass pieces (for scanning electron microscopy).

Conditions of Irradiation

The cells were harvested for irradiation at 10–14 days after seeding when they had formed a stable, confluent monolayer and were in a state of topoinhibition of cell division and movement.

The cultures were exposed to λ radiation generated by 8 MeV electrons in a linear accelerator (MEL SL 75 Super). The radiation dose was 200 Gy given at a dose rate of 17 Gy/min (Hamberg *et al.* 1976).

The growth medium was, usually immediately following irradiation, replaced by conditioned medium obtained from control cultures in which dense cells had been cultivated for two days.

Selection of Observation Periods

Since earlier studies have shown that cytoplasmic and membranous alterations were fully developed within 4 days after irradiation, and that no alterations appreciable by SEM or light microscopy occurred before 6 hours after irradiation (Hamberg *et al.* 1976) cells were fixed for SEM and light microscopy at intervals varying from 6 to 96 hours after irradiation. To detect early changes of cell and plasma membrane motility time-lapse cinemicrography was continued from 1 to 72 hours after irradiation.

Phase Contrast Microscopy

The cells were fixed in 2% glutaraldehyde in 0.1 M Na-cacodylate-HCl buffer with 0.1 M sucrose (pH 7.2, 510 mOsm) (Collins *et al.* 1977a). Following fixation the cells were allowed to remain in the fixative solution or were dehydrated in ethanol and mounted with Eukitt. Observations were made and photographs were taken by use of a standard phase contrast photomicroscope equipped with a phase contrast water immersion or an oil immersion objective lens (both × 40). Photographs were also taken with a × 63 oil immersion lens of living, unfixed cultures mounted in the Dvorak Stodler chamber.

Time-lapse Cinemicrographs

The 25 mm glass coverslips were mounted in a Dvorak Stodler chamber into which growth medium was injected. The medium from the Petri dishes in which the cells had grown was used. The films were made on a Zeiss photomicroscope (No. III) using a phase plane, panchromatic λ 40 oil-immersion objective lens (N.A. 1.0) and a 100 mm ocular. The camera was a Bolex Pallard 16 mm controlled by a Wild MBF B control unit and a MBF-G timer. The film speed was 20 frames/min., which was considered adequate to permit a detailed study of ruffling movements of the plasma membranes. Each field of view was followed for about 3.5 hours. Utilizing the maximal number of exposures in each strip of film (4,200 frames). Only one randomly selected area of each culture was filmed. The equipment was placed in a walk-in incubator at 37° C.

Scanning Electron Microscopy

Cells were prepared for SEM at 6, 12, 24, 48 and 96 hours after irradiation. The cells were fixed in 2% glutaraldehyde in 0.1 M Na-cacodylate HCl with 0.1 M sucrose (pH 7.2, total osmolality 510 mOsm vehicle osmolality 300 mOsm) (Collins *et al.* 1977a). The fixation was initiated by slowly adding 2.5 ml of the fixative, pre-warmed to 37° C to the dishes containing 2.5 ml of the medium. Care was taken not to move the dishes or change their pH, or temperature, before fixation, since ruffles have proved to be sensitive structures which easily withdraw when the cells are disturbed (Blomquist *et al.* 1978). After five minutes, the medium fixative mixture was sucked off, pure fixative at 37° C was added and the fixation continued for another 10 min at 37° C. The cultures were then removed from the incubator and the fixation continued in a refrigerator for at least another 45 min.

The cells were post fixed in 1% OsO₄ in 0.15 M Na-cacodylate-HCl and dehydrated in a graded series of acetone solutions, starting with 50%. The cells were critical point dried from CO₂ in a Polaron E 3000 apparatus. The specimens were gold-sputtered in a Polaron E 5000 sputter at about 0.1 torr, 12 kV and 70 mA providing a 300 Å thick gold layer. The cultures were studied in a Jeol JSM I microscope at 10 kV or in a Jeol 100-C transmission electron microscope, with scanning attachment, at 40 kV. Micrographs were taken with the specimens tilted 45°.

Fig 1 Phase contrast micrograph of control culture composed of flat gila cells with rounded nuclei and abundant cytoplasm. Glutaraldehyde fixation, oil immersion objective lens × 480

Fig 2 24 hours after irradiation. Numerous dark folds (arrows) representing ruffling membranes are superimposed on the contours of a nucleus. Phase contrast micrograph of unfixed culture in a Dvorak Stodler chamber oil immersion objective lens × 970

Fig 3 24 hours after irradiation. A small ruffle of a tripolar appearance (arrow) is situated on the upper cell surface adjacent to the nucleus. The picture is suggestive of vacuolar formation in association with the ruffle. Other cells contain clusters of large vacuoles (V). Phase contrast micrograph of glutaraldehyde fixed cells. Water immersion objective lens × 590

Fig 4 42 hours after irradiation. Large clusters of vacuoles are present in many of the cells. Glutaraldehyde fixed cells water immersion objective lens × 480

Fig 5 18 hours after irradiation. Numerous ruffles (arrows) originating in the contact zones between closely packed cells are illustrated. Unfixed cells. Water immersion objective lens × 480

Fig 6 48 hours after irradiation. Phase contrast micrograph illustrating vacuolated cells with severe degenerative changes and partial dissolution of the cytoplasm. Glutaraldehyde fixed cells, oil immersion objective lens × 960



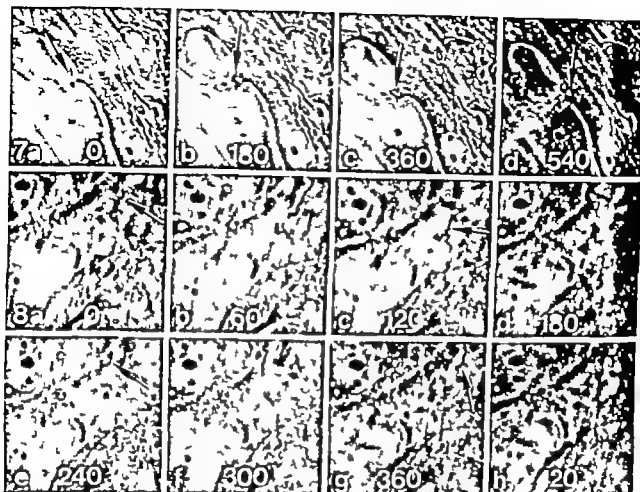


Fig 7 24 hours after irradiation. A sequence from a time lapse film showing a ruffle forming in a cell that overlaps a neighbouring cell and gives rise to a cluster of endocytotic vacuoles (arrow). The time is indicated in seconds.

Fig 8 40 hours after irradiation. Time lapse sequence of a crowded culture with ruffling membranes (arrows) occurring in an apparently disorganized fashion with no formation of endocytotic vacuoles during the time of observation.

RESULTS

Light Microscopy and Time-lapse Cinemicrography

Controls 10–14 days after seeding the cultures formed a dense tissue-like monolayer composed of flat cells with a rounded nucleus and abundant cytoplasm (Fig 1). The peripheral parts of the cytoplasm were frequently overlapped but nuclear overlapping was observed only in small areas where cells piled up in a fashion described earlier (Westermarck, 1971). Mitoses were extremely uncommon. Single or small groups of vacuoles were observed only in a few cells, and peripheral ruffling of plasma membranes was present only in small areas where cell contact was not absolutely tight. Ruffles arising from the upper cell surface were never encountered.

Irradiated cells. From approximately 12 hours after irradiation, occasional cells showed an elongated, astrocyte-like appearance with slender cytoplasmic

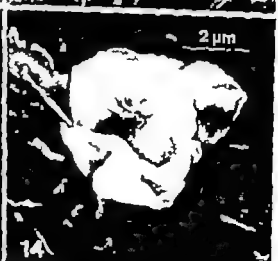
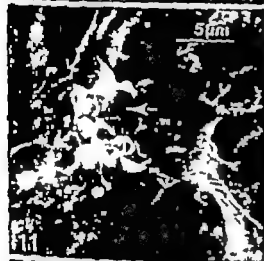
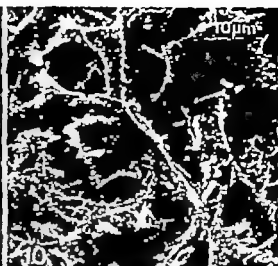
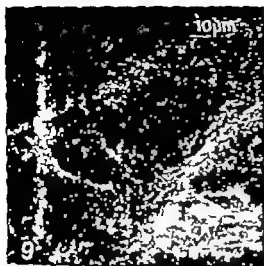
Fig 9 Scanning electron micrograph (SEM) of a control culture. The cells are closely packed. The surfaces are flat with a moderate amount of microvilli.

Fig 10 96 hours after irradiation. The cells are retracted and partly of a star shaped appearance. Microvilli are scanty.

Fig 11 18 hours after irradiation. Peripheral ruffling in an area of close cell contact. A caveolar formation, probably representing an endocytotic vacuole, is seen at the base of the ruffling membrane (arrow).

Fig 12 48 hours after irradiation. A partially collapsed balloon-like structure on the cell surface. The picture is probably representing the SEM correlate of the blebbing that could be observed by light microscopy.

Figs 13 and 14 6 hours after irradiation. Two different appearances of atypical ruffles arising on the upper cell surface far from the borders of any neighbouring cells.



mic projections. Some of the cells were of a bipolar spindle-like appearance; others were star-shaped with three or more slender projections. The number of thus altered cells increased during the following days, and three days after irradiation more than 50% of the cells were altered (Hamberg *et al* 1976). Concomitantly occasional dying cells were seen to loosen from the plastic dish and float freely in the growth medium.

Approximately 6–12 hours after irradiation, ruffling of cell membranes was obviously increased in the irradiated cells, as compared to controls. At the 24 hour interval ruffling was even more intense, occurring in at least 50% of the cells in one field of view during 35 hours observation. Ruffles appeared as a dark rim with numerous folds. Some ruffles were of a small type, also occurring at the cell borders of a limited number of control cells (Fig. 7).

The major γ of the ruffles, however, consisted of a rather broad fan-shaped upwardly directed undulating membrane. These ruffles (that were not encountered in densely grown control cells) occurred rather often at more than one side of the cell border simultaneously (Fig. 8). The ruffles were seen to emerge at the contact zone between closely packed cells (Fig. 5) or even to arise from a part of cytoplasm overlapping another cell (Fig. 2).

In a small number of cells, ruffles also seemed to occur from the upper cell surface. These so-called ruffles were of a varied appearance, some were of a circular type, whereas others consisted of short, cytoplasmic foldings (Fig. 3). Ruffles were often seen adjacent to or overlapping nuclei. Only in rare instances was any kind of cell debris seen on the cell surface in the vicinity of central ruffling membranes.

Ruffling remained on a rather high level at 48 hours and then gradually decreased during the next 2 days. At 72 hours, augmented plasma membrane motility was merely seen in contracting or dying cells. These cells showed a period of intense ruffling and blebbing during which the cytoplasm loosened from its peripheral attachments and contracted to the star-shaped or bipolar appearance. The contraction phase was generally completed in 10–30

minutes. Following contraction, the cell membranes became quiet and motionless in some cells, the cells still adhering to the plastic dish surface. In some of the observed cells, however, blebbing of the cell membrane increased in magnitude and intensity; the cells rapidly acquired a rounded appearance, apparently died, and finally lost contact with the petri dish surface.

Enhanced endocytosis appeared concomitantly with increased ruffling. Thus, there was a slight increase of endocytotic vacuoles at 6–12 hours after irradiation (Table 1). At the 24 hour interval clusters of four or more macropinocytotic vesicles were visible in approximately 30% of the cells (Fig. 4). Some cells showed a great part of the cytoplasm occupied by such vesicles. During the next 2 days, endocytotic vacuoles gradually decreased in number but still occurred even in severely degenerated cells (Fig. 6). The contracted, star- or spindle-shaped cells, however, contained only occasional vacuoles.

Some vacuoles seemed to arise at the cell border in connection with ruffling membranes in the way that macropinocytotic vesicles are ordinarily created (Fig. 7). In a few instances, vacuolar formation appeared to occur in connection with atypical, central ruffling from the upper cell surface (Fig. 3). In a great number of cells containing numerous vacuoles, no evidence of membrane ruffling was found during the period of cinematographic observation.

Scanning Electron Microscopy

Controls The cultures consisted of closely packed cells with flat surfaces, that were smooth except for a variable amount of slender microvilli (Fig. 9). Cytoplasmic blebs or tiny ruffles at the cell borders in areas of incomplete cell contact were rarely encountered. Occasional cells displayed small concave like or pits, probably representing endocytotic vesicles. Star-shaped or bipolar shrunken cells were infrequent.

Irradiated cells Elongated, astrocyte-like cells with slender projections appeared in increased amounts from approximately 12 hours onwards. These cells often displayed a number of fine retraction fibers running from the cell border to the original points of attachment to the plastic dish surface. The shrunken and elongated cells usually showed very few microvilli on the cell surface (Fig. 10).

Increased ruffling was observed from the 12 hour interval after irradiation. The ruffles were mostly of the peripheral type, arising from a thin leading lamella that very often overlapped the cytoplasm of an adjacent cell (Fig. 11). The ruffles consisted of thin upwardly directed membranous

TABLE 1 Percentage of Cells with More than 3 Vacuoles per Cell. Approximately 700 Cells Studied in Each Group in a Phase Contrast Microscope

	6 hrs	12 hrs	18 hrs	24 hrs	48 hrs
Controls	4%	7%	6%	7%	7%
Irradiated	4%	25%	29%	78%	18%

sides in some cells, however small linear or arular membranous folds seemed to arise from the upper cell surface far from the borders of any overlapping cytoplasm (Figs. 13 and 14). Dead cells or parts thereof were usually not encountered in association with these ruffles. Partially collapsed balloon-like membranous structures were moderately frequent (Fig. 12).

An increased number of cells were found to contain small pits on the surface. In many instances these remnants of endocytotic vesicles were found in association with ruffling membranes at the periphery of the cells (Fig. 11).

DISCUSSION

The characteristic findings in the X-irradiated cultures were retraction and rounding up of the cells, start of both peripheral and central ruffling activity and a pronounced endocytosis associated with the ruffling membranes. The latter phenomena reached their maximum after about 24 hours, while retraction and rounding up of the cells appeared to occur with great variability in time and represent a stage preceding severe degeneration and cell death.

With regard to the augmented endocytosis and ruffling activity previous TEM studies had revealed increased numbers of small, often plasma membrane-associated, vesicles or macropinocytotic vacuoles of presumed endocytotic nature (Hamberg *et al.* 1977). Although large vacuoles were also encountered relatively often in these cells, it was difficult with the TEM to obtain quantitative data supporting the action of an increase of such vacuoles. The vacuoles observed with time-lapse cinematography evidently were of the large variety. Some of these large vacuoles were shown in the present study to be created directly at the level of the plasma membrane in ruffling areas. In the previous report (other) large vacuoles appeared to be created by the fusion of small ones. Together the two studies indicate that irradiation of glia cells results in a greatly enhanced diffuse macropinocytosis and a macroendocytosis associated with ruffling areas. The combined effect of these endocytotic activities evidently is the internalization, and possible degradation, of a considerable plasma membrane surface.

The factors and mechanisms which govern different types of endocytosis are incompletely understood. Perhaps best known are the effects of exogenous compounds present in the culture medium. For instance, a large variety of molecules and chemical substances have been shown to be inducers of endocytosis (Chapman-Andersen 1965, Cohen and Parts 1967, Gordon 1973). Since there is no change in the chemical composition of the medium used

before or after irradiation, and since the control cultures showed a low endocytotic activity, exogenous factors can evidently be ruled out as inducers of the augmented endocytosis.

As summarized in a previous study the significance of the increased endocytosis could be: (a) purposeful augmented uptake of nutrients from the growth medium, (b) internalization and degradation of pieces of injured or otherwise altered plasma membranes, (c) diminution of the total cell surface, (d) supplementation of membrane material to autophagic processes in the cytoplasm. It is also possible that the change is governed by radiation-induced damage to the cytoskeleton in particular the microtubules and the microfilaments, or central regulatory mechanisms in the nucleus.

In our model, the given dose is high and no cells survive longer than about 10 days. Furthermore, no mitotic activity recurs and the cells synthesizing mechanisms are likely to be severely damaged. Since the active ruffling and associated pinocytosis results in internalization of large amounts of plasma membrane and the cells turn very narrow and thread-like, the replacement or *de novo* synthesis of membranes is probably not operating or is at least greatly impaired.

Ruffling and associated phenomena were first described by Lewis (1931), and these phenomena have been carefully studied, mainly on macrophages and amoebae, but also on various types of *in vitro* cultured cells. Apart from being of importance for endocytosis, ruffling has been related to cell locomotion and also found to be correlated with cell multiplication (Harris 1973, Schellens *et al.* 1976, Bruuk *et al.* 1977). The importance of ruffling and associated pinocytosis for cell multiplication is unclear. Both uptake of nutrients and turnover of plasma membrane might be events necessary for the cell during the completion of a cell cycle. It is noteworthy that normal cells under standard culture conditions only show peripheral ruffling activity when growing in sparse cultures, while many malignant cell lines show both peripheral and central ruffling activity even in crowded cultures (Collins *et al.* 1977b, Borek and Fenech 1976). Central, atypical ruffling has also been observed on normal, diploid glia cells during the process of heterophagocytosis of cell organelles (Collins *et al.* 1976). However we did not find evidence of heterophagocytosis in connection with the atypical ruffling of the irradiated glia cells.

Our finding of vigorous ruffling on density inhibited G₁ blocked glia cells which do not divide suggests that enhanced such activity may occur as a result of cell or plasma membrane damage, possibly during subsequent regenerative efforts.

This leads to the disappearance of a large part of the plasma membrane, resulting in the branched, threadlike cells found in an irradiated culture. The diminished cell surface area indicates the creation, following irradiation, of an unbalance between degradation and renewal of the plasma membrane.

Supported by grants from the Swedish Medical Research Council (B78-12X-01006-13A) and the Swedish Cancer Society (Project No. 617 B78-07ab).

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MORPHOLOGICAL FEATURES IN NON-CIRRHOTIC LIVERS FROM PATIENTS WITH CHRONIC ALCOHOLISM, DIABETES MELLITUS OR ADIPOSITAS

A Comparative Study

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Christoffersen, P. & Petersen, P. Morphological features in non-cirrhotic livers from patients with chronic alcoholism, diabetes mellitus or adipositas. A comparative study. Acta path. microbiol. scand. Sect. A, 86: 495-498, 1978.

Consecutive liver biopsies from alcoholic, diabetic and overweight patients are compared morphologically and in addition a comparison is made between groups with a combination of two or three of the above conditions. Both fatty change and morphological activity are greater in the groups with alcoholism, and this gives good reason to believe that the activity in the form of alcoholic hepatitis is the cause for the more common development of cirrhosis in alcoholic fatty liver than in fatty liver with other aetiology.

Key words: Liver morphology, alcoholism, diabetes mellitus, adipositas.

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Received 4 x 78 Accepted 24 x 78

Fatty liver is often seen in patients with chronic alcoholism, diabetes mellitus or overweight (10-11) (1), but it is well-known that it is the alcoholic fatty liver which most often proceeds to cirrhosis.

The purpose of the present paper has been to compare the incidence and degree of different histological qualities in a consecutive material of liver biopsies from patients with chronic alcoholism, diabetes mellitus, or overweight in order to evaluate whether there are morphological variables or combinations of variables which may explain why alcoholism leads to cirrhosis more frequently than diabetes or overweight.

MATERIAL AND METHODS

During the period 1964-1969 percutaneous liver biopsies have been performed by the method of Mengesha in all cases with clinical and/or laboratory signs of liver

diseases; in patients with chronic alcoholism (consumption of more than five drinks per day for more than two years), untreated non-insulin-requiring diabetes and in overweight conditions (body weight greater than 110 per cent of standard weight) as described previously (8).

Biopsies with cirrhosis (defined by parenchymal nodules and fibrosis), suspicion of cirrhosis, signs of cholestasis of chlorpromazine type or extrahepatic cholestasis, signs of viral hepatitis, malformation, neoplasm, and vascular disorders were excluded, and the material for this investigation comprises 99 biopsies.

The tissue has been fixed in neutral formalin and embedded in paraffin. The biopsies were 1-1.4 cm thick and 1.3-4.5 cm long and were cut as serial sections (6 µm - 57 sections).

The assessment has been performed by one of the authors (P.C.) without any knowledge of the clinical data on haematoxylin and eosin and v. Gieson-Hauser stained sections. Furthermore, additional sections from all biopsies stained for reticulin fibres (6), iron (7) and pyroninophil substance (1) have been available.

The occurrence of the following qualities has been registered. Mallory bodies (2), alcoholic hepatitis (4), focal necroses (liver cell necroses excluding necroses in alcoholic hepatitis and lipogranulomas), Kupffer cell proliferation, cholestasis (incontrovertible intra- or extra-cellular bile thrombi) lipogranulomas (3) parenchymal inflammation, bile duct proliferation, and inflammation of the portal tracts, as well as iron deposits in liver cells, Kupffer cells and connective tissue. Further the degree of fatty change and fibrosis has been registered.

Fatty change is quantified in the following manner

- 0 the biopsies contain no fat vacuoles
- +
- the biopsies contain fatty vacuoles, but on average in less than one third of the liver cells. Included as slight fatty change have also been biopsies which only contain a few lipid droplets in each lobule.
- ++
- the biopsies contain fatty vacuoles in one third or more of the cells but in less than two thirds of the cells.
- +++
- the biopsies contain fatty vacuoles in two thirds or more of the cells.

The 99 patients comprising the material were divided into seven groups according to three factors - alone or combined chronic alcoholism, diabetes mellitus, and overweight (conf. table 1). The histological features of the groups have been compared.

For the statistical evaluation the χ^2 - test has been used and the limit for type I error (2 α) has been placed at 0.05

RESULTS

A summary of the parenchymal changes in the biopsies from the seven groups of patients is given in Table 1

Fatty change occurs more often and more pronounced in the biopsies from »pure» alcoholics than in biopsies from »pure» diabetics and »pure» overweight, and the difference is significant ($p < 0.01$) in comparison with the overweight group. In addition, fatty change occurs more often when diabetes and overweight respectively are connected with alcoholism than in the groups of »pure» diabetics and »pure» overweight. The combination of diabetes and overweight with or without simultaneous alcoholism determines the highest frequency of fatty change. The fatty change is in all cases distributed diffusely in the lobules but in some cases with a slight centrilobular dominance.

Focal necroses are observed in the majority of biopsies from alcoholics, and there are significantly more biopsies with necrosis in the alcoholic group (18 (82 per cent)) than in the diabetic group (2 (17 per cent)) ($p < 0.001$) and the overweight group (3 (43 per cent)) ($p < 0.05$). The combination of alcoholism with either diabetes or overweight determines a higher frequency than in the »pure» groups. In only two of the biopsies, both from the alcoholic group, are many necroses found. In all cases they are distributed diffusely throughout the parenchyma. Confluent necroses and piecemeal necroses have not been demonstrated.

TABLE 1 Number of Biopsies with the Following Parenchymal Changes from 92 Patients with Alcoholism (A), 12 Patients with Diabetes (D), 7 Patients with Overweight (O), 71 Patients with Diabetes and Overweight (D+O), 8 Patients with Diabetes and Alcoholism (D+A), 19 Patients with Overweight and Alcoholism (O+A) and 10 Patients with Diabetes, Overweight and Alcoholism (D+O+A)

Parenchymal changes		A (22)	D (12)	O (7)	D+O (21)	D+A (8)	O+A (19)	D+O+A (10)
Fatty change	slight	15 (68%)	7 (58%)	3 (43%)	10 (48%)	6 (75%)	12 (63%)	7 (70%)
	moderate	3 (14%)	0 (0%)	0 (0%)	9 (43%)	1 (13%)	5 (26%)	2 (20%)
	severe	1 (5%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (10%)
Focal necroses		18 (82%)	2 (17%)	3 (43%)	12 (57%)	5 (63%)	10 (52%)	8 (80%)
Lipogranulomas		7 (32%)	0 (0%)	1 (14%)	7 (33%)	3 (38%)	4 (21%)	5 (50%)
Mallory bodies		3 (14%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (20%)
Alcoholic hepatitis		2 (9%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Kupffer cell proliferation		16 (73%)	3 (25%)	1 (14%)	9 (43%)	5 (63%)	6 (32%)	8 (80%)
Inflammation		11 (50%)	0 (0%)	1 (14%)	6 (29%)	4 (50%)	8 (42%)	8 (80%)
Cholestasis		0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Iron content		5 (23%)	3 (25%)	1 (14%)	3 (14%)	2 (25%)	3 (16%)	2 (20%)

The figures indicate the number of biopsies in each subgroup of the nine qualities. The percentage which the number constitutes of the subgroup is given in brackets.

TABLE 2 Number of Biopsies in the Following Portal Changes in the Seven Groups of the Material

Portal changes	A (22)	D (12)	O (7)	D+O (21)	D+A (8)	O+A (19)	D+O+A (10)
slight	7 (5%)	0 (0%)	2 (29%)	1 (5%)	1 (13%)	1 (5%)	1 (10%)
moderate	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
severe	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Bile duct proliferation	1 (5%)	0 (0%)	1 (14%)	0 (0%)	0 (0%)	0 (0%)	2 (20%)
Inflammation	5 (23%)	1 (8%)	2 (29%)	9 (43%)	2 (25%)	6 (32%)	5 (50%)

The figures indicate the number of biopsies in each subgroup of the three qualities.

The percentage which the number constitutes of the subgroup is given in brackets.

Lipogranulomas are found most often in biopsies from alcoholics and diabetics in connection with overweight. The combination of alcoholism with diabetes or with overweight is connected with a higher frequency of lipogranulomas than in the sparse groups of diabetes or overweight. In all cases the lipogranulomas are small and located centrilobularly. In addition all the biopsies show fatty change. There is no significant difference in the occurrence of lipogranulomas in the different groups.

Mallory bodies have been found in three biopsies in the alcoholic group and all three biopsies show some degree of fatty change. Two of the three biopsies in addition show changes as in alcoholic hepatitis with necrosis of liver cells containing Mallory bodies and infiltration with neutrophils in the surroundings.

Microbial reaction in the parenchyma. Both the incidence of hepatic cells proliferation ($p < 0.05$) and of parenchymal inflammatory cells ($p < 0.05$) are greater in the group from alcoholics than in the other two sparse groups, and the combination with alcoholism determines a higher frequency than in the sparse group.

Cholestasis and iron content. Only a minority of the biopsies exhibit iron deposits and none of the biopsies exhibit cholestasis.

A summary of the changes in the portal tracts is given in Table 2. All the changes are slight, only found in few biopsies, and without significant difference between the groups.

DISCUSSION

Fatty liver is a common finding in both alcoholics, overweight patients and diabetes, but occurs more often and more pronounced in alcoholics.

It is a well-known observation that many persons with fatty liver develop fibrosis and eventually

cirrhosis, but that in other instances even severe steatosis may be present for years without development of cirrhosis (9). It is in addition well-known that it is the alcoholic fatty liver which most often proceeds to cirrhosis, and in this connection the question may be asked: Has the present study shown morphological disparities which may explain this difference?

In liver with fatty change one may in the light of the microscope observe liver cells disintegrating in the following manners: focal liver cell necrosis, lipogranulomas and necrosis in alcoholic hepatitis. If the histological activity in a fatty liver is expressed by the number of disintegrated liver cells and the degree of inflammatory reaction brought about by these, there is in the presented material a greater activity in the groups of biopsies from patients with a history of alcoholism than in the other groups, and this fact tends to confirm the assumption, that alcohol not only induces alcoholic hepatitis but also the other kinds of necroses (5).

Follow-up investigations render it probable, that focal necroses and small lipogranulomas generally disappear without sequelae and can thus not be the link between fatty change and fibrosis (3).

Mallory bodies and alcoholic hepatitis are only seen in the group of alcoholics, and as previously shown, parenchymal fibrosis is more pronounced in biopsies with fatty change (without cirrhosis) and Mallory bodies than in biopsies with fatty change (without cirrhosis) and no Mallory bodies (5).

It is therefore very likely that the answer to the above question is that alcoholic hepatitis is the common cause for the more frequent development of cirrhosis in alcoholic fatty liver than in fatty liver from other causes.

Our thanks are due to Professor Torben Schmidt, M.D. Department of Pathological Anatomy, Rigshospitalet, for lending us the biopsy material.

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CLINICAL RELEVANCE OF HISTOLOGICAL GRADING OF CANCER OF THE LARYNX

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Helweg-Larsen, K., Grem, N., Meistrup-Larsen, K.-I. & Meistrup-Larsen, U. Clinical relevance of histological grading of cancer of the larynx. *Acta path. microbiol. scand. Sect. A* 86 499-504 1978.

Primary biopsies from 52 patients with cancer of the larynx were examined by two pathologists working independently. The malignancy of each case was graded twice with an interval of 1-1½ month according to a histological scoring system elaborated by Jacobsson. The reproducibility of the system was found poor for individual prognostic purposes and no connection between histological grade and clinical course was demonstrated.

Key words: Cancer of the larynx, histological grading, reproducibility.

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Received 31.1.78; Accepted 6.v.78

Grading of malignant tumours is prognostically relevant only if there is a clear connection between the ascribed degree of malignancy and the clinical course. Moreover the grading must be reproducible by different pathologists and by the same pathologist at any time.

Recently Jacobsson (1973a, 1973b) described a scoring system for evaluation of epidermoid carcinomas of the larynx. This system was modified and used by Lund et al. (1975a, 1975b, 1976, 1977) in grading carcinomas of lip, tongue and larynx. The grading system was recommended as a decisive factor in the choice of therapy.

We have applied this system on a series of 52 patients with cancer of the larynx. The purpose was to estimate the reproducibility of the method and the connection between the grade and the clinical course.

MATERIAL AND METHOD

Patients. The series consists of 52 patients: 42 men and 10 women. Sex distribution and age at the time of diagnosis are shown in Fig. 1. All patients were primarily seen at the Department of Oncology, Hørrningshospitalet, Copenhagen during the period 1969-1973. One in five biopsies were performed by direct laryngoscopy. The histological diagnosis was in all cases epidermoid carcinoma, including cases of severe dysplasia with possible invasion. Cases with carcinoma in situ were omitted. 22 carcinomas were localized in the supraglottic, 29 in the glottic, and 1 in the subglottic area. The series was classified according to the TNM-system (UICC 1974) (Table 1).

TABLE 1 Distribution of the 52 Cases According to TNM Classification (UICC 1974)

T1a	18	N0	49	50	52
T1b	4	N1	1		
T2	18	N2	0		
T3	3	N3	2		
T4	4				

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Treatment

45 patients were primarily treated by radiation (Cobalt - experimental). 3 patients in addition had a total laryngectomy due to persistent stenosis by tumour after radiation. In 2 patients total laryngectomy was performed without previous radiation. 2 patients had only local excision of the tumour. A total laryngectomy was

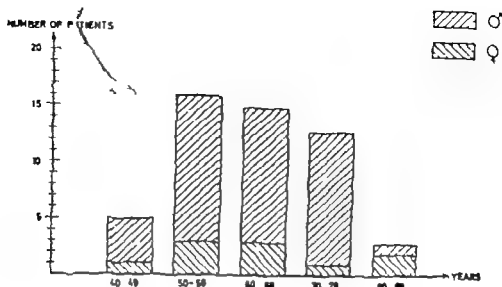


Fig 1 Age groups and sex distribution

performed in 9 of 24 patients with local recurrence. In the remaining 15 patients high age, complicating illness or metastases were contraindication to laryngectomy.

Clinical Course

In the clinical evaluation local recurrence (verified by biopsy) metastases in regional lymph nodes (clinical judgment) and lethality due to cancer of the larynx were considered in an observation period of 3 years (Table 2).

Histology

The histologic grading system is as described by Jacobsson (1973a, 1973b) (Table 3). The system is based upon registration of 8 morphological parameters, each graded in a four stage scale. The score values of the 8 parameters are summarized in some biopsies all 8 parameters are not evaluable, therefore all carcinomas are characterized by histological score, defined as the

total sum of points divided by the number of parameters assessed (Lund *et al* 1975a).

The original sections of the initial biopsies were used for the histological grading. The biopsies were all fixed in formalin, embedded in paraffin and cut in step sections average 3 μ m in thickness from 9 different levels. We used haematoxylin-eosin stained slides for the evaluation.

Study of Reproducibility

Without knowledge of former histological description two of the authors, P₁ (NG) and P₂ (hlf), blindly graded the biopsies twice, analysis I and II with an interval of 1-1 month. The interpersonal reproducibility was assessed by comparing pairs of histological scores from the two pathologists. This was done both in analysis I and II. The intrapersonal reproducibility was assessed by comparing pairs of histological scores from the two analyses. This was done for both of the pathologists.

Statistical Analysis

The means of histological scores in the 4 gradings for patients with and without local recurrence, regional lymph node metastases and death due to cancer of the larynx were compared. The reproducibility of the grading was evaluated by double readings of biopsies and subsequent calculations of standard deviation of histological scores, expressed by the formula:

$$s^2 = \frac{1}{2n} \sum d^2$$

where s = standard deviation, n = number of histological gradings and d = observed differences (Therkelsen 1976).

TABLE 2 Recurrence Metastases in Regional Lymph Nodes and Mortality Due to Cancer of the Larynx

+ Local recurrence	74 pts
- Local recurrence	78 pts
+ Metastases in regional lymph nodes	12 pts
- Metastases in regional lymph nodes	39 pts
+ Metastases in regional lymph nodes	1 pt
Death due to carcinoma laryngis	33 pts
Alive after 3 years	15 pts
Death due to other causes	4 pts

TABLE 3 *Histological Grading of Malignancy*

Parameter number	Tumour cell population	Points			
		1	2	3	4
1	Structure	Papillary and solid	Strands	Small cords and groups of cells	Marked cellular disassociation
2	Differentiation	Highly keratinization	Moderately some keratinization	Poorly Minimum keratinization	Poorly no keratinization
3	Nuclear polymorphism	Few enlarged nuclei	Moderate number of enlarged nuclei	Numerous irregular enlarged nuclei	Anaplastic immature enlarged nuclei
4	Masses	Single	Moderate number	Great number	Numerous
	Tumour-host relationship	Points			
		1	2	3	4
5	Mode of invasion	Well defined borderline	Cords less marked borderline	Groups of cells no distinct borderline	Diffuse growth
6	Stage of invasion	Possibly	Microcarcinoma (few cords)	Nodular into connective tissue	Massive
7	Vascular invasion	None	Possibly	Few	Numerous
8	Cellular response (plasmocytosis, pleocytosis)	Marked	Moderate	Slight	None

RESULTS

Correlation between Histological Scores and Clinical Data

4 analyses of the biopsies were performed by the two pathologists. None of the four analyses showed any correlation between the means of histological scores and local recurrence, metastases in regional lymph nodes, and mortality due to cancer of the breast (Table 4).

Reproducibility

The possible maximum of paired parameters in the 4 analyses are 416 (52×8). It was possible to

evaluate 413, 403, 404 and 411 pairs of parameters in the 4 analyses (Table 5). Agreements - disagreements in the scores of the individual parameters are given next in Table 5. Same score was found in 301, 235, 204 and 209 pairs of parameters, a difference of one point was found in minimum 107 and maximum 178 pairs of parameters. Difference of 3 points was found only in 3 and 2 pairs.

By the above mentioned formula standard deviations for the histological scores were calculated (Table 6). The two first numbers read from left to right thus indicate the degree of accuracy of the individual pathologist, (P_1 0.20 P_2 0.37) and the last two numbers indicate the degree of accuracy in

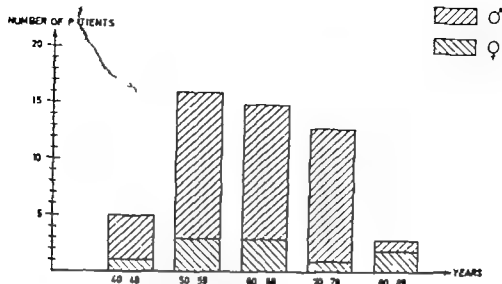


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The original sections of the initial biopsies were used for the histological grading. The biopsies were all fixed in formalin, embedded in paraffin and cut in step sections average 5 μ m in thickness from 9 different levels. We used haematoxylin-eosin stained slides for the evaluation.

Study of Reproducibility

Without knowledge of former histological descriptions two of the authors, P₁ (N.G.) and P₂ (K.H.), blindly graded the biopsies twice (analyses I and II) with an interval of 1-1 month. The interpersonal reproducibility was assessed by comparing pairs of histological scores from the two pathologists. This was done both in analysis I and II. The intrapersonal reproducibility was assessed by comparing pairs of histological scores from the two analyses. This was done for both of the pathologists.

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$$s^2 = \frac{1}{2n} \sum d^2$$

where s = standard deviation, n = number of histological gradings and d = observed differences (Therrelsen 1976)

TABLE 2 Recurrence Metastases in Regional Lymph Nodes and Mortality Due to Cancer of the Larynx

+ Local recurrence	74 pts
- Local recurrence	28 pts
+ Metastases in regional lymph nodes	17 pts
- Metastases in regional lymph nodes	39 pts
* Metastases in regional lymph nodes	1 pt
Death due to carcinoma laryngis	33 pts
Alive after 3 years	15 pts
Death due to other causes	4 pts

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Parameter number	Tumour cell population	Points			
		1	2	3	4
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4	Mitoses	Scanty	Moderate number	Great number	Numerous
	Tumour-host relationship	Points			
		1	2	3	4
5	Mode of invasion	Well defined borderline	Cords, less marked borderline	Groups of cells no distinct borderline	Diffuse growth
6	Stage of invasion	Possibly	Microcarcinoma (few cords)	Nodular into connective tissue	Massive
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TABLE 4 *Correlation between the Clinical Data in the Observation Period of 3 Years and the Means of the Histological Scores Given by the Two Examiners in the First and the Second Analysis*

Clinical data		Local recurrence				Metastases in regional lymph nodes				Death due to cancer of the larynx			
		+		-		+		-		+		-	
Analysis		I	II	I	II	I	II	I	II	I	II	I	II
Means of histological scores	P ₁	2.3	2.4	2.3	2.5	2.5	2.6	2.3	2.4	2.5	2.5	2.2	2.4
	P ₂	2.2	2.2	2.0	2.3	2.3	2.7	2.1	2.7	2.2	2.1	2.1	2.2

TABLE 5 *Total Numbers of Evaluated Paired Parameters and the Distribution of the Numbers According to Differences in Their Score Values*

		P ₁ (I + II)	P ₂ (I + II)	I (P ₁ + P ₂)	II (P ₁ + P ₂)
Total numbers		413	403	404	411
Number with difference in score values	Difference 0	301	235	204	209
	1	107	143	165	178
	2	7	23	33	22
	3	3	7	2	2

TABLE 6 *Intra- and Interpersonal Reproducibility Expressed as Standard Deviations of Histological Scores*

	P ₁ (I + II)	P ₂ (I + II)	I (P ₁ + P ₂)	II (P ₁ + P ₂)
%Histological score	0.70	0.37	0.37	0.37

TABLE 7 *Standard Deviations of Histological Scores with Score Values < 2.5 and ≥ 2.5*

	P ₁ (I + II)	P ₂ (I + II)	I (P ₁ + P ₂)	II (P ₁ + P ₂)
%Histological score < 2.5	0.15	0.39	0.3	0.35
%Histological score ≥ 2.5	0.23	0.33	0.37	0.22

the two analyses (I 0.32 and II 0.35). The material was divided into groups with histological score < 2.5 and with histological score ≥ 2.5 (Table 7).

DISCUSSION

In grading biopsies from 52 patients with cancer of the larynx we found no relation of the histological score to the clinical course. This is in contrast to

series of Jacobsson (1973a, 1973b) and of Lund *et al* (1976, 1977), who both found evident relationship. This discrepancy can be explained by the fact that our series is small and that the series consists of relatively more women, older patients and supraglottic localisations than seen in the other materials. But contributory factors can be difference in biopsy and preparation procedures.

Even small biopsies from a lesion of the larynx

are usually adequate for simple diagnostic purposes. This does not imply that the biopsies are representative for the tumour and adequate for purposes of grading which demands multiple and bigger biopsies to minimize sample error.

The histological technique in our and previous works was not standardized. Like Jacobsson (1973a, 1973b) and Lund *et al* (1976, 1977) we used the original slides. The time from biopsy to fixation of the tissue, number and thickness of sections, cut per biopsy are uncertain factors.

Reproducibility

We found it correct to use the simple statistical method for calculation of standard deviations of histological scores. The method demands, that the standard deviations are the same all over the scale. We found this demand met, (table 7). The slight differences can be explained by random variation due to the low number of patients in the series. A better accordance in grading was not found in the second analysis. This might have been expected. The persistent variation, in spite of training, reflects the difficulties in exact interpretation of the system.

In the following we have examined the difficulties in grading the individual parameters. We emphasize that the reason for choice of the eight parameters and their possible overlap is not discussed in this context.

In general we did not find a 4 range scale suitable for evaluation of all the individual parameters and have by testing the system learned that a distinction in many of the parameters between grade 2 and 3 is difficult.

Structure and mode of invasion, parameter 1 and 5 are well defined and easily recognized.

Differentiation, parameter 2 is evaluated by the degree of keratin formation. Jacobsson (1973a, 1973b) and Lund *et al* (1975a, 1975b, 1976, 1977) do not define, neither if it is the procentual number or area of keratinized cells which is registered, nor if the assessment should be equally based on superficial or deep parts of the biopsy. The latter point is important because of a frequent difference between the amount of keratin in the superficial and the deep layers of tumours. Our assessments are based upon equal evaluation of keratinized areas in superficial and deep parts of biopsies.

Nuclear polymorphism, parameter 3 is by Jacobsson (1973a, 1973b) evaluated by estimating numbers of enlarged nuclei. We did not find this definition applicable, but used the modification by Lund *et al* (1975a, 1975b, 1976, 1977), who

applied the classification of Broders (1926, 1958) and assessed the procentual incidence of mature nuclei.

Mitosis, parameter 4 was by Jacobsson (1973a, 1973b) graded as single, moderate number great number or numerous mitoses. Lund *et al* (1975a, 1975b, 1976, 1977) counted mitoses per microscopic field at a magnification of 250 in at least 5 fields and thus tried to make the criteria more precise. The type of microscope i.e. the size of microscopic field is not indicated, nor whether to base the count on most mitose-active fields or on average active fields.

Many factors may influence the number of mitoses such as delay in fixation, (Evans 1926), tension of oxygen (Bulbough 1950). Silherberg (1976) tested the interpersonal reproducibility of six experienced pathologists in counting mitoses, and found it poor especially because the criteria for mitoses are not distinct.

For evaluating the number of mitoses we used Jacobsson's criteria which we found reasonable. We have used the presence of distinguishable chromosomes as our criterion for mitoses and have omitted pyknotic or hyperchromatic nuclei.

Stage of invasion, parameter 6 was not difficult to estimate in grade 1 and 2 but as the biopsies often were superficial with tumour infiltration to the bottom of the biopsy it was impossible to distinguish between grade 3 and 4. Lund *et al* (1976) used the relation of tumour to submucosa as a criterion. This tightening of the criteria concerning invasion is though not usable, due to the wellknown hesitation in defining a submucosa in the larynx. (Wiburn 1964). As we did not find the criterion «massive» applicable, we have chosen to regard invasion in either skeletal muscles or in perichondrium as grade 4.

Vascular invasion, parameter 7 In theory we do not find evaluation of invasion into vessels difficult, but we doubt the value of this parameter as there were only few and very small vessels in our biopsies. In contrast to Lund *et al* (1975) we did not find it possible to distinguish between lymph vessels and blood vessels of so small size.

Cellular response, parameter 8 is defined by easily applicable criteria. But thickness of sections is also here of importance. In our scoring the total inflammatory cellular response is evaluated even though an ulcerated surface may cause an extensive inflammatory reaction on the basis of infection.

Clinical Relevance

In previous studies (Jacobsson 1973a, 1973b; Lund *et al.* 1975a, 1975b, 1976, 1977) an indication of degree of accuracy was not given, which is imperative to evaluate the prognostic statement.

The accuracy in our study expressed by standard deviations varies between 0.20 and 0.37—7–12% of the 1–4 scale. Other investigators of semiquantitative scoring systems have found an accuracy of the same magnitude. Champion and Wallace (1971) thus found in grading carcinoma of the breast an interpersonal disagreement of 30% using a 3 stage scale. In their series as a whole they both found a good and identical correlation of grade and prognosis. Variations in grading have little influence on clinicopathological correlations in large groups and histological grading is practicable and of value in analysis of the natural history of cancer and its response to treatment.

But allocation of a numerical value to the histopathology of a particular tumour does not imply prognostic precision for the individual case.

CONCLUSION

In testing a system of histological grading we found no correlation to the clinical course of disease in 52 patients. The assessed reproducibility was of the same magnitude as found by other investigators of semiquantitative scoring systems. Whether the tested grading system is clinically relevant, i.e. able to distinguish between patients with good and bad prognosis and thereby useful as a basis for therapeutic measures is still to be proven.

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RAPID PLATELET CONSUMPTION IN A CASE OF METASTATIC OSTEOGENIC SARCOMA OF THE BREAST

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Johansson, S., Kutti, J. & Olsson, L.-B. Rapid platelet consumption in a case of metastatic osteogenic sarcoma of the breast. *Acta path. microbiol. scand. Sect. A*, 86: 505-511 1978.

An operation was carried out on an elderly female because of an osteogenic sarcoma of the breast. One year later she developed a state of rapid thrombocytolysis resistant to high dose prednisolone therapy and splenectomy. At autopsy a large single metastasis was found in the right atrium and tumour emboli in the pulmonary artery. It is suggested that the shortening in platelet survival might have had an immunological background. Trapping of fibrous material within tumour emboli and mechanical platelet destruction caused by the tumour appear to have been contributing factors.

Key words: Osteogenic sarcoma, heart, thrombocytopenia.

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Received 6.1.78 Accepted 31.1.78

Osteogenic sarcoma of the soft tissue is rare (Sjostedt & Larss 1967, Allen & Somle 1971) and the published examples of osteogenic sarcoma of the breast are less than 200 (Sawick & Taylor 1969). Extracerebral osteogenic sarcoma occurs in older age groups and are localized mainly in the deep parts of the extremities (Sjostedt & Larss 1967). The prognosis is poor and the patients die because of disseminated metastatic disease (Wernstrom *et al* 1963, Sjostedt & Larss 1967).

The present communication deals with a patient with metastatic osteogenic sarcoma in whom thrombocytopenia was a major clinical problem. Platelet survival was shown to be very short, and it is suggested that the rapid platelet consumption might have had an immunological background, although other factors may have contributed.

CASE REPORT

A 67-year-old woman was admitted on October 19, 1975 to the Department of Medicine, Sahlgren's Hospital, Göteborg, because of chest pain and shortness of breath.

In November 1974 she had undergone radical mastectomy with axillary node dissection because of a cytologically verified malignant tumour of the left breast. The tumour was round and poorly circumscribed, measuring 2 cm in diameter. It was hard and bony and was classified as an osteogenic sarcoma. The axillary lymph nodes were negative for tumour growth. The patient received post-operative irradiation.

On admission, physical examination was unremarkable except for mild tachycardia. There were no signs of increased bleeding tendency from the skin or mucous membranes. A chest X-ray showed moderate enlargement of the heart. The ECG and the enzyme studies were all negative. After admission to hospital the chest pain disappeared but returned on October 29. A friction rub was now heard upon auscultation, and the ECG showed signs of pericarditis. The patient improved after the institution of prednisolone.

Normal values for the peripheral platelet count had been recorded in November 1974 as well as in April 1975 but on admission it was noted that the patient was thrombocytopenic, with a platelet count of 20,000/ μ l. The haemoglobin value was 11.5 g/100 ml and WBC 8,100/ μ l. The clinical course from admission to death is shown in Fig. 1. Other laboratory findings were: ESR 34 mm/h, RBC 3.4×10^6 / μ l and reticulocytes 4.4%. Direct and indirect Coombs tests were negative. No cold

Clinical Relevance

In previous studies (Jacobsson 1973a, 1973b Lund *et al* 1975a, 1975b 1976 1977) an indication of degree of accuracy was not given, which is imperative to evaluate the prognostic statement.

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TABLE 1 Results of the duplicate platelet survival studies. The control values have been published elsewhere (Branchfiel *et al.* 1974)

Platelet survival study	Platelet mean life-span (days)	Platelet production rate ($\times 10^{10}$ platelets/days)
December 5 (autologous platelets)	0.5	44
December 9 (homologous platelets)	0.1	67
Controls: Neon	6.9	22
SD	1.3	6

samples were drawn at 5, 15, 30, 45, 60, 120 minutes and 20 hours after infusion. The subsequent samples were obtained at 24-hour intervals. After differential centrifugation the platelet-bound radioactivity was analysed in a well-type scintillation counter. Platelet PLIS was derived from the zero-time slope of the platelet survival curve (Kurtz & Hensfield 1971; Branchfiel *et al.* 1974). This was achieved by fitting a gamma-function (Murray & Francis 1977) to the experimental data by the least squares method, using a digital computer (Branchfiel *et al.* 1975). Platelet production rate was obtained from N/PLIS , where N denotes the total number of circulating platelets (Kurtz & Hensfield 1971; Kurtz & Hensfield 1972; Kurtz & Sefton-Kane 1975).

Histological methods. 5 μ sections were stained with haematoxylin-eosin, Weigert van Gieson, Masson-Trichrome, Alcian blue at pH 0.5 and 2.5 and toluidine blue at pH 0.5 and 4.0. Special stain for fibrin (Ladenberg) was also performed.

PATHOLOGICAL ANATOMY

Autopsy Findings

At autopsy the patient's skin showed multiple petechiae. Status after mastectomy was noted. The heart was enlarged and weighed 590 g. The normal outline of the heart was distorted (Fig. 2) because of a large, solid, oval tumour measuring 7.5 cm



Fig. 2 Distorted outline of the heart due to a large tumour mass in the wall of the right atrium.

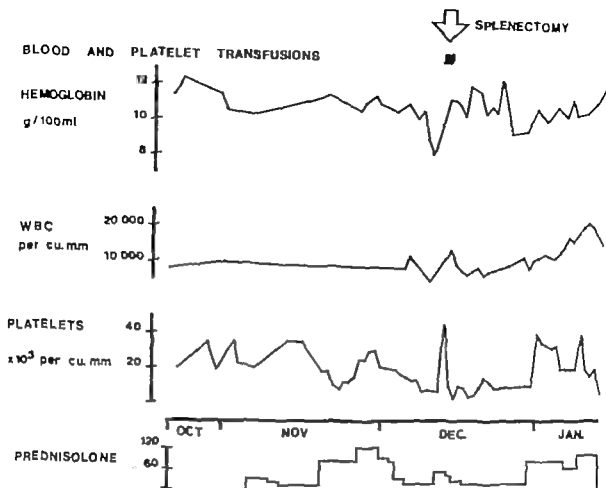


Fig 1 Course of disease and treatment

agglutinins were detected in the serum. Antinuclear antibodies were not present. On several occasions the levels of factor VIII, fibrinogen and fibrinogen degradation products were within normal limits. The differential count of sternal bone marrow smears was normal. Histological examination of biopsy specimens from spinal processes showed a normal amount of megakaryocytes. No splenic enlargement was present on flat plate X ray. The platelet count remained low (Fig 1) and the patient developed easy bruising with purpura and petechiae on mucous membranes and skin. It was considered that the thrombocytopenia could be due to a rapid peripheral platelet destruction induced by a viral infection. However complement fixation tests for adenovirus, Coxsackie B, cytomegalovirus, herpes simplex, influenza A and B, ornithosis, morbilli, mycoplasma pneumoniae, rubella and toxoplasma were all within normal ranges. No antiplatelet antibodies could be detected by the method described by Karpatkin & Saklad (1969).

On November 5 30 mg/day prednisolone by mouth was instituted. Though the dose was subsequently increased to 70 mg/day no rise in the peripheral platelet count occurred (Fig 1). Platelet survival studies were carried out on December 5 and December 9. In the first experiment autologous, and in the second homo-

gous, ^{51}Cr -labelled platelets were employed. The results are summarized in Table 1. On both occasions the platelet mean life span (MLS) was shown to be considerably shortened and platelet production slightly increased. Splenectomy was considered to be indicated and was performed on December 12. On the day of surgery the patient received transfusions of platelets as well as packed red cells (Fig 1). The spleen weighed 74 g and histological examination reported no noteworthy pathological changes. Post-operatively no rise in the peripheral thrombocyte count occurred. At the beginning of January 1976 she developed a liver condition with increasing levels of bilirubin, alkaline phosphatase, SGOT and SGPT. The condition deteriorated rapidly and death took place on January 13.

METHODS

Platelet kinetics. Duplicate platelet survival studies were carried out, once using autologous and the second time homologous platelets from an ABO-Rh-compatible donor. The platelets were labelled *in vitro* with ^{51}Cr and were thereafter injected into the patient. The technique employed has been described in detail elsewhere (Kutis & Hensfield 1971; Furti & Scharf-Kutis 1975). Blood



Fig 4 Cut surface of the hard part of the heart tumour



Fig 5 The opened right chamber (RC) of the heart and the pulmonary artery (PA) showing multiple tumour emboli (TE)



Fig 6 Section from the hard part of the heart tumour



Fig 7 Section from the breast tumour showing highly



Fig 3 Opened right heart showing polypoid extension of the tumour (PE), TV = tricuspid valve.

growing in the wall of the right atrium. The solid tumour mass extended to the tricuspid valve and from there a polypoid extension measuring 5×1.5 cm was hanging like a pendulum down through the opening of the tricuspid valve into the right chamber of the heart (Fig. 3). The large, solid tumour mass was firm and the cut surface was greyish white and fleshy (Fig. 4). The pendulum-like part of the tumour was soft and gelatinous, and the cut surface was greyish-green. The right chamber was partly filled with several large gelatinous tumour emboli. The right pulmonary artery was totally occluded by gelatinous tumour emboli. The branches of the left pulmonary artery extending to the upper lobe and lingula were likewise occluded by gelatinous tumour emboli (Fig. 5). No further tumour spread was found. The branch of the right pulmonary artery supplying the median lobe was peripherally occluded by an embolus, and a 5×5 cm wedge-shaped haemorrhagic infarct was found in the lung parenchyma. The cut surface of the liver showed signs of severe passive and acute congestion.

Histological Findings

Histologically the tumour varied somewhat in different areas. Both the breast tumour and the heart tumour were composed of highly pleomorphic spindle-shaped cells interlaced with areas of malignant osteoid (Fig. 6). Bone formation was prominent, particularly in the breast tumour (Fig. 7). Mitoses were frequent. The gelatinous part of the cardiac tumour as well as the tumour emboli were composed of atypical chondroid cells. Both the surface of the polypoid extension of the tumour and the endocardial area adjacent to the tricuspid valve were covered with fibrinous material to a large extent. The hard part of the tumour contained quite a lot of collagen fibres, but fibrinous material was not found within this part of the tumour. However, the tumour emboli contained substantial amounts of fibrinous material. On histological examination the haemorrhagic infarct was verified in the lung. Multiple emboli were seen in several smaller branches of the pulmonary artery in the right median lobe close to the area of infarction. No other infarcted areas were detected in the lungs.

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DISCUSSION

The mammary tumour in the present study fulfils the criteria for osteogenic sarcoma of soft tissue as described by *Stout & Lattes* (1967) i.e. 1) the presence of a uniform morphological pattern of sarcoma that excludes the possibility of mixed malignant mesenchymal tumour 2) the production of sarcomatous tissue of malignant osteoid or bone (or both) and 3) the ready exclusion of osseous origin. The tumour in this case exhibited highly pleomorphic spindle-shaped cells focally associated with atypical osteoid and bone, as well as areas of chondroid differentiation. Bone and cartilage may be seen in other breast tumours, namely «mixed tumours» (histologically identical with mixed tumours of salivary gland) cystosarcoma phylloides and adenocarcinoma with metaplasia (*Smith & Taylor* 1969) the latter being a possible differential diagnosis. However in the latter case there is always a transition from carcinoma to heterotopic element (*Smith & Taylor* 1969) and this was not found in the present case.

The breast tumour is considered to be primary and the cardiac tumour metastatic. This may be supported by a time interval of more than one year between diagnoses of the two tumours. Metastases to the heart are rare (*Herbut & Maisel* 1942) and are seen most commonly in patients with disseminated malignant disease from primaries in the lungs, breasts and oesophagus, often as a direct extension of the tumour (*Scott & Garvin* 1939). To our knowledge only two cases of primary osteogenic sarcoma of the heart have so far been described in the literature (*Lown & McInee* 1972, *Lasin* 1974) one of which was associated with widespread metastases. The present cardiac tumour was located in the right side of the heart, which has been described to be the predominating side in other cases of cardiac metastases (*Prichard* 1951, *DeLoach & Haynes* 1953). It is notable that, besides the cardiac tumour, no further tumour spread was found in the present case. Solitary metastases to the heart appear to be exceedingly rare, and cardiac metastases are almost invariably associated with disseminated disease (*Scott & Garvin* 1939).

Thrombocytopenia was a major clinical problem during the course of the disease. The results of platelet survival studies demonstrated clearly that a state of rapid platelet consumption was the underlying cause of the low peripheral platelet concentration. Platelet MLS was shown to be dramatically shortened and within the range seen in patients with very active forms of idiopathic thrombocytopenic purpura (ITP) (*Branchög et al.* 1974). Thus, when autologous platelets were employed, the MLS for

the circulating platelets was calculated to be 0.5 day. In the experiment with homologous platelets VLS was only 0.1 day (Table 1). However since the platelet count at the time of study was only about 10 000/ μ l, the results obtained with homologous cells must be considered to be more representative of the true survival of the circulating platelet population. In agreement with what is seen in ITP the platelet production rate was slightly increased (2–3 times) (*Harker* 1970, *Branchög et al.* 1974). There were no laboratory signs of disseminated intravascular coagulation. Using the platelet factor 3 immunoinjury test (*Karpatkin & Siskind* 1969), no platelet antibodies could be detected. However as recently demonstrated by us, the value of this technique in the detection of antiplatelet antibodies is questionable (*Kutti & Safai-Kutti* 1978). Necropsy disclosed a large tumour mass in the right atrium with a fibrin-covered polypoid extension hanging through the opening of the tricuspid valve into the right chamber. Fibrinous material was also found trapped within the tumour emboli but not within the main tumour mass. Emboli were found in the branches of the pulmonary artery supplying the median lobe of the right lung, which was partly infarcted, but there was no evidence elsewhere in the lungs of repeated episodes of embolization.

It seems reasonable to assume that the shortening of platelet survival could be attributed mainly to a similar mechanism which governs the rapid thrombocytolysis in ITP. The peripheral destruction of platelets was very intense, since neither corticosteroids nor splenectomy alleviated the thrombocytopenia.

In view of the necropsy findings, trapping of fibrinous material within the tumour emboli and mechanical platelet destruction appear to have contributed to the platelet consumption.

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A RETROSPECTIVE HISTOLOGICAL STUDY OF 669 CASES OF PRIMARY CUTANEOUS MALIGNANT MELANOMA IN CLINICAL STAGE I

The Relation of Cell Type, Pigmentation, Atypia and Mitotic Count to Histological Type and Prognosis

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Larsen, T. E. & Grude, T. (1978). A retrospective histological study of 669 cases of primary cutaneous malignant melanoma in clinical stage I. The relation of cell type, pigmentation, atypia and mitotic count to histological type and prognosis. *Acta path. microbiol. scand. Sect. A*, 86: 513-522, 1978.

A selected series of 669 primary malignant melanomas of the skin, stage I, was studied. The series includes 86 lentigo maligna melanomas, 259 superficial spreading malignant melanomas, 194 nodular malignant melanomas and 130 undifferentiated malignant melanomas. The tumour cell type was classified and the tumour cell pigmentation, the cellular atypia and the mitotic count was graded. The relation of these four tumour cell features to each other and to the tumour type was studied by χ^2 -tests. The prognosis, also of these features in relation to the total series as well as to each tumour type was also examined. The most common features were mixed cellularity, little pigment, moderate atypia and low mitotic count. Most of these tumours were superficial spreading malignant melanomas. A good prognosis was related to spindle-shaped tumour cells, marked pigmentation, slight atypia and few mitoses. A bad prognosis was related to epithelioid tumour cells, little pigment, marked atypia and many mitoses. Variations of lentigo maligna melanoma tended to be more benign while variations of nodular malignant melanoma tended to be more malignant than the average. A superficial spreading malignant melanoma might vary in either direction.

Key words: Melanoma, cell type, pigmentation, atypia, mitotic count, prognosis.

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Received 70.11.78 Accepted 5.1.78

We reported in a previous paper (Larsen & Grude 1978) the method of selection of 669 primary malignant melanomas (MM) of the skin in clinical stage I (256 men and 413 women). The sections were classified according to Clark's system (Clark 1967) without access to any clinical information. We found 86 lentigo maligna melanoma (LMM), 259 superficial spreading malignant melanoma (SSM), 194 nodular malignant melanoma (NMM), and 130 undifferentiated malignant melanoma (UMM).

The histological classification of MM proposed by Clark (1967) pays no attention to characteristics of the tumour cells such as morphological type, pigmentation, atypia and mitoses. It was therefore found of interest to study the prognostic importance of these features independently of the tumour type and thereafter to look at their connection with each of the types hoping to be able to explain why these have a significantly different prognosis (Larsen & Grude 1978).

The features studied in this paper are only some of the parameters which are important for the

reports of MM Hermsævi *et al.* (1976) found that depth of invasion is of a greater prognostic importance than tumour type. Thus and other prognostic important parameters such as ulceration, pigmentation of the surface, vascular invasion and lymphocyte reaction have also been studied. The result of these investigations will be treated in following papers.

MATERIALS AND METHODS

The tumours studied the specimens separately without access to clinical information. Our individual results were compared later. Agreement was reached in most cases. When this was impossible and when poor material quality of the section or heavy pigmentation such as the grading of one or several factors impossible, the factor was coded as indeterminate. Information concerning the histological typing and the survival of the patients was given later by the Cancer Registry of Norway.

Tumour Cell Type

We classified the cells as epithelioid (Fig. 1), small lymphocyte-like (Fig. 1) and spindle shaped (Fig. 2). The presence of more than one cell type led to a classification of mixed cellularity. We hoped thereby to keep the 3 main groups as pure as possible. Conradsson *et al.* (1974) found that patients with MM formed of small epithelioid cells have a significantly poorer prognosis than those with MM made of big epithelioid cells. We did not grade the epithelioid cells.

Tumour Cell Pigmentation

Pigmentation was graded as 0 (amelanotic), +, ++ and +++ Grade 0 included cases with no pigment granules in the cytoplasm of the tumour cells by using a magnification of 400 \times but no special staining for melanin (Clark 1969). Not even the slightest pigmentation of the adjacent, non-oncotic part of the tumour was

allowed. Cases with slight to moderate pigmentation of part of the tumour were classified as grade + while grade ++ included cases in which all available parts of the tumour were slightly or moderately pigmented. Cases with a localized or diffuse heavy pigmentation of the tumour were classified as grade +++.

Atypia

This factor was graded +, ++ or +++ according to the highest degree which was found. We classified cells resembling benign junctional or dermal melanocytes as grade + (Fig. 1 and 2) and anaplastic cells as grade +++ (Fig. 4). Cells which could not be placed in either of these groups were classified as grade ++ (Fig. 1 and 3) (McGovern 1970). At the same time we took notice of the size of the nucleoli (Fig. 3 and 5) which appears to vary with the grade of atypia (Lurie 1972) and with the prognosis of ocular melanomas (Caldwell *et al.* 1942).

Mitotic Count

The number of mitotic figures were counted in 5 high power ($\times 400$) fields (in the most dedifferentiated areas of large tumours) and graded as follows: grade + ~ 0-1 mitoses/5 high power fields, grade ++ ~ 2-4 mitoses/5 high power fields and grade +++ ~ 5 or more mitoses/5 high power fields. This is approximately the same as recommended by McGovern *et al.* (1973). In doubtful cases of grade + or ++ 5 additional fields were examined and the greatest number of mitotic figures were used for grading.

RESULTS

The Grading/Classification and the Prognostic Value of Tumour Cell Type, Pigmentation, Atypia and Mitotic Count

Tumour cell type. The relative frequency of the different cell types appears from Fig. 6. Most cases show a mixed cellularity (357 ~ 53%). No tumours were found consisting only of small, lymphocyte-like cells.

The observed cumulative survival rates (Cancer Registry of Norway 1975) have been arranged according to the different cell types and are also shown in Fig. 6. The survival of patients with epithelioid celled MM appears to be less than of those with spindle celled MM. The difference is not significant, probably because the group of spindle celled tumours is small. The survival of patients with tumours showing a mixed cellularity is significantly better than that of patients with epithelioid celled MM after 10 years. When each tumour type is considered separately this significant difference disappears. When each tumour cell type is considered, the significant difference in survival of the various tumour types disappears except with regard to the difference between SVM and NMM with epithelioid cells at 10 years after diagnosis.

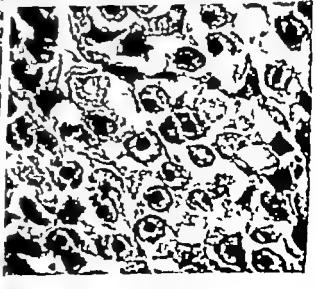
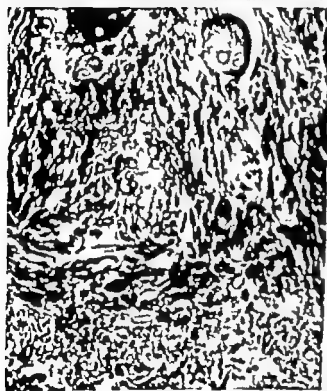
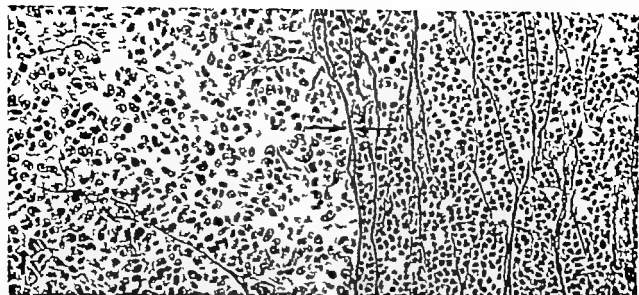
Fig. 1 To the left large epithelioid tumour cells with atypia grade +. (a) To the right (sharply demarcated from them) small epithelioid tumour cells especially those (b) and lymphocyte-like tumour cells especially those (c) with additional border-line cell types, all types with atypia grade -. From a NMM 195.

Fig. 2 Spindle shaped tumour cells with atypia grade +. From a LMM $\times 195$.

Fig. 3 Size of nucleoli corresponding to atypia grade. Border-line cell type between epithelioid and spindle cell. From a SVM 540.

Fig. 4 Giant epithelioid tumour cells with atypia grade. From a melanotic MM $\times 340$.

Fig. 5 Size of nucleoli corresponding to atypia grade +. Small epithelioid tumour cells. From a SVM $\times 750$.



Mitotic Count

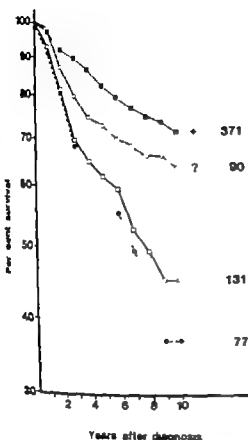


Fig 9 Survival curves illustrating the observed cumulative survival rates of patients having tumours with different grades of mitotic count

much shorter than of those with grade +. The difference in the survival rates is significant at 5 and 10 years after diagnosis, also between grade + and + and at 10 years even between grade + and +.

When looking at only SMIM a significant difference in the survival rates is present between grade + and + and at 10 years after diagnosis even between grade + and +. There are no LMM with grade + of atypia and only 7 NMIM with grade +. The difference in the survival rates of cases with LMM showing grade + and + of atypia and of those with NMIM showing grade + and + is not significant. Not versa, when only one grade of atypia is considered the significant difference in the survival of the 3 tumour types disappears except with regard to those with grade +.

Mitotic count Most tumours (371 ~ 55.5%) showed only few mitoses. Fig. 9 illustrates the observed cumulative survival rates of our patients related to the number of mitotic figures in their MM. The patients with a high mitotic count have the worst prognosis. The difference between grade + and ++ or +++ is significant both at 5 and 10 years after diagnosis. This is so even in all types of MM except NMIM after 5 years. There is, however, no cases of LMM with many mitoses and only 4 cases with grade ++ of mitotic count. Concerning cases with SMIM and NMIM and grade ++ or +++ of mitotic count the difference in survival is significant after 10 years but not after 5 years. When we look at cases with few mitoses only there is a significant difference in survival between the 3 tumour types at 5 and 10 years after diagnosis, except between LMM and SMIM after 5 years.

The Relationship between the Different Features and between These and the Tumour Type

Table 1 shows the result of χ^2 tests concerning these various relationships. There is a strong tendency of the indeterminate grades/types to be linked to each other. When evaluating the χ^2 value, we have therefore eliminated these groups without changing the degrees of freedom. The obvious trends concerning the relationship between the determinate grades/types appears from the table.

DISCUSSION

When trying to interpret the importance of the various features studied in this paper to the prognosis of the patients with different tumour types we have looked at 1) the prognostic value of the various grades/types of each feature independently of tumour type, 2) the relation of these grades/types to the other features considering their prognostic value and 3) the relation of the various grades/types of each feature to tumour type.

When comparing the relation of one feature to two others (e.g. the tendency of NMIM to show marked atypia and a high mitotic count) we have usually not checked the overlapping of the individual cases.

Concerning the results of the χ^2 tests (Table 1) one ought to remember that a significant χ^2 value only proves that the two features which have been compared do not vary independently of one another and not that they are dependent of each other. They may both be closely linked to one and same (third) feature. Further a high significance may be due to

Cell type

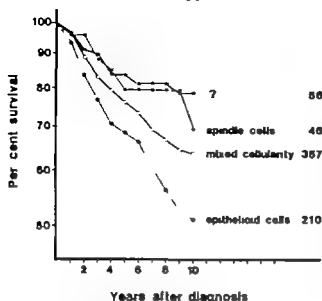


Fig 6 Survival curves illustrating the observed cumulative survival rates of patients having tumours made of different cell types.

Pigmentation Most of our MM showed pigmentation grade + (387 ~ 58%), (Fig. 7).

Further Fig 7 illustrates the observed cumulative survival rates of MM with different degrees of pigmentation. Grades ++ and +++ have a better prognosis than grades + and 0. At 5 and 10 years after diagnosis the survival of patients with amelanotic MM is significantly worse than of those showing grade ++. This difference disappears, however if only NMM is considered, and in the case of SVM it is present at 5 years after diagnosis, not after 10 years. There are no amelanotic LMM. The significant difference in the survival of patients with various tumour types is still present when only cases showing grade ++ of pigmentation are considered. There is, however no significant difference between the survival of the patients with amelanotic SVM and amelanotic NVM.

Atypia Fig 8 shows the distribution of the different degrees of atypia. Most of the tumours (370 ~ 55%) showed moderate atypia.

The observed cumulative survival rates of the patients showing different grades of cellular atypia in their MM are further illustrated in Fig. 8. The survival of patients with marked cellular atypia is

Pigmentation

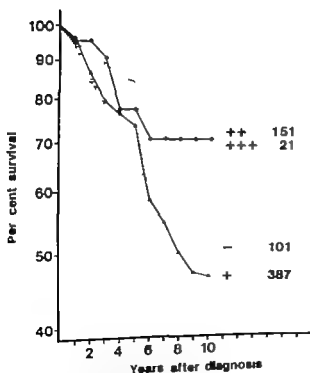


Fig 7 Survival curves illustrating the observed cumulative survival rates of patients having tumours with different grades of pigmentation. The survival of 9 cases with indeterminate grade of pigmentation is not illustrated.

Atypia

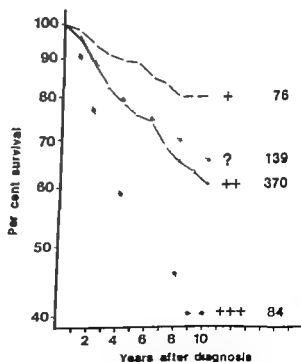


Fig 8 Survival curves illustrating the observed cumulative survival rates of patients having tumours with different grades of atypia.

port of another cell type with diverging grade of pigmentation, atypia and/or mitotic count (Fig. 1). This may indicate the growth of different cell clones (Gray & Perce 1964 Clark *et al* 1969 Klemm 1970 Fidler & Kripke 1977). We are aware that the total number of sections from each tumour may lead to a wrong classification of a few NM with mixed cellularity as tumours with only one cell type.

The relative frequency of the different cell types in our series (Fig. 6) is difficult to compare with that of other investigators partly because of their small series (Hardmeier *et al* 1968), and partly because the authors do not separate tumours of pure cell type from those of mixed cellularity (Cochran 1969 Hardmeier *et al* 1970 Gartmann & Trusch 1972). Our results may be comparable with those of Gerrier & Thormann (1968) who found the same low frequency of spindle celled tumours as in our series.

We have not found any references to the relative frequency of the different cell types in each of the various types of NM, except that Clark *et al* (1969) mentioned that all the 3 types of NM may show epitheloid cells, spindle cells and small cells, and that there is a tendency for spindle cells to be common in LMN and epitheloid cells to be most common in SMN. This is in agreement with our findings (Table 1) as regards the spindle cells, while the epitheloid cells seem to be correlated equally strongly with SMN and NMN in our series. Castermans *et al* (1974) mention that spindle cells are frequent in LMN but infrequent in the other NM. This is also in agreement with what we have found concerning the LMN and SMN. Spindle cells are, however, not infrequent among the NMN in our series.

There is a weak tendency of spindle cells to show slight atypia. In fact, no spindle cells with marked atypia were found. This may explain the relatively good prognosis of our patients with spindle celled tumours (Fig. 6). Most other authors did not find any significant relationship between cell type and prognosis (for further references see Knutson *et al* 1971 Gartmann & Trusch 1972 Little 1972). Our findings are however in agreement with those concerning melanomas of the choroid and ciliary body which show the same cell types as the cutaneous NM (Caldwell *et al* 1942).

The findings concerning cell type is difficult to interpret. They may explain partly why patients with LMN have a better prognosis than those with the two other types of NM but not why patients with NMN have a shorter survival than those with

Pigmentation

It soon became evident that this factor was difficult to grade. Most of the tumours showed a very uneven distribution of pigment, sometimes together with mixed cellularity as mentioned earlier. This phenomenon has already been described by other investigators (Thormann *et al* 1970 Clark *et al* 1975).

It is difficult to compare the relative frequency of our different grades of pigmentation (Fig. 7) with that of other investigators (Hardmeier *et al* 1968 Cochran 1969 and Beardmore *et al* 1970) because the grading methods are not the same. We have tried to extract two groups of NM (grade 0 and ++-) which were as homogeneous as possible, the one including cases in which apparently all tumour cells produce melanin and the other amelanotic. We thereby hoped to make it reasonable to compare the survival of these two groups. We are aware that the pigmentation perhaps is not homogeneous throughout the whole tumour in some cases because we have only got a limited number of sections from each case.

Many investigators have failed to demonstrate any correlation between the pigmentation of NM and survival (for further references see Hines 1974 Chung *et al* 1975 Elias *et al* 1977). This may be because they have not compared homogeneous groups. However the histological findings of Hardmeier *et al* (1968) and Little (1972) and the clinical findings of Sjöhem (1949) correspond with our results which show a better prognosis for the more pigmented tumours (Fig. 7). Furthermore, our findings fit in well with the significant tendency for amelanotic tumour cells in our series to show marked atypia and/or be rich in mitoses and/or to form NMN while moderate pigmentation seems to be associated with less atypia and/or fewer mitoses and/or LMN (Table 1). Our finding that the grade of pigmentation varies inversely with the mitotic count is in agreement with the findings of Gray & Perce (1964) in transplanted NM cell-lines and with those of Schockschabel (1971) and Kreider *et al* (1975) in NM cell-cultures.

Provided that the grade of pigmentation indicates the differentiation of the tumour cell (Beardmore *et al* 1970), the highly significant pattern in the relation between pigmentation and tumour type may partly explain the difference in the prognosis of the patients with various tumour types.

Atypia

The grade of atypia may vary from one area to another in a single tumour (Fig. 1) as mentioned earlier. We used the most atypical cells for grading even when they were present only in a small part of

TABLE 1 The Relationship between Tumour cell Type Pigmentation Atypia Mitotic Count and Tumour Type

Features compared	(N)	N	P	Obvious trends among the determinate grades and types
Cell type/pigmentation	(81/96)	3/31	>0.95	0
Cell type/mitotic count	(43/04)	6/30	>0.1	0
Cell type/atypia	(166/14)	15/32	>0.05	(Spindle cells ~ + atypia)
Pigmentation/atypia	(100/67)	24/41	<0.01	0 pigment ~ +++ atypia ++ pigment ~ + atypia
Cell type/tumour type	(53/26)	40/25	<0.001	Spindle cells ~ LMM Epithelioid cells ~ NMM
Pigmentation/mitotic count	(69/00)	48/77	<0.001	0 pigment ~ +++ mitotic count ++ pigment ~ + mitotic count
Atypia/tumour type	(60/94)	51/67	<0.001	+ atypia ~ LMM +++ atypia ~ NMM
Pigmentation/tumour type	(71/92)	61/08	<0.001	0 pigment ~ NMM ++ pigment ~ LMM (SMM)
Atypia/mitotic count	(136/97)	63/44	<0.001	+ atypia ~ + mitotic count +++ atypia ~ +++ mitotic count
Mitotic count/tumour type	(115/44)	99/89	<0.001	+ mitotic count ~ LMM +++ mitotic count ~ NMM

The indeterminate grades and types eliminated

9 degrees of freedom

The complete tables are available from the author

the linkage of some uninteresting factors as some of the indeterminate features in the present series. We have therefore been interested in the existence of a certain trend in the relationship between the determinate features only.

We cannot exclude the possibility that our present impressions will be changed when the result of the multiple regression analysis is available.

Tumour Cell Type

Romsdahl (1968) described 3 different types of MM cells in cell cultures, i.e. cuboid, spindle-shaped and dendritic, and Hakini (1975) grew two different cell types, i.e. 'polygonal or spheroidal' and 'elongated, fusiform'.

The existence of different cell types in MM is not surprising in view of the wide morphological spectrum of normal epidermal melanocytes as described by Beardmore *et al.* (1976), assuming that these melanocytes are the histogenetic source of the proliferating tumour cells of all 3 types of MM (Clark 1967). If one prefers the theory that the neoplastic cells of a benign naevus are the mother

cells at least of SMM and NMM (Mishima 1967), then there are several types of these: A- B- and C cells (Miescher & Albertini 1935) or epithelioid, lymphocytelike and neurod cells, which in fact correspond to the main cell types of MM.

When attempting to classify cell type, we often had problems due to technical artefacts such as shrinkage and/or compression. In other cases, however, it was obviously due to border-line cell types between small epithelioid cells and lymphocyte-like cells (Fig. 1) and between epithelioid cells and spindle-shaped cells (Fig. 3). Besides, it was sometimes difficult to distinguish small epithelioid or lymphocytelike MM cells from benign small melanocytes which made it difficult to decide whether there was a co-existent benign naevus or not. Other investigators have had the same problem (Little 1972; Reed *et al.* 1975).

In the mixed cellularly group epithelioid cells were often mixed with spindle cells. In some few cases all three types of cell were found in one tumour. Sometimes we found domination of one cell type with the well-demarcated additional

growth of another cell type with diverging grade of pigmentation, atypia and/or mitotic count (Fig. 1). This may indicate the growth of different cell clones (Gray & Pierce 1964 Clark *et al.* 1969 Kleemann 1970, Fisher & Krjale 1977). We are aware that the limited number of sections from each tumour may have led to a wrong classification of a few MM with mixed cellularity as tumours with only one cell type.

The relative frequency of the different cell types in our series (Fig. 6) is difficult to compare with that of other investigators partly because of their small series (Hardmeier *et al.* 1968), and partly because the authors do not separate tumours of pure cell type from those of mixed cellularity (Cochran 1969 Beardmore *et al.* 1970 Gartmann & Tritsch 1972). Our results may be comparable with those of Gerber & Thormann (1968) who found the same low frequency of spindle celled tumours as in our series.

We have not found any references to the relative frequency of the different cell types in each of the various types of MM except that Clark *et al.* (1969) mentioned that all the 3 types of MM may show epithelioid cells, spindle cells and small cells, and that there is a tendency for spindle cells to be common in LMM and epithelioid cells to be most common in SMM. This is in agreement with our findings (Table 1) as regards the spindle cells, while the epithelioid cells seem to be correlated equally strongly with SMM and NMM in our series. Casassina *et al.* (1974) mention that spindle cells are frequent in LMM but infrequent in the other MM. This is also in agreement with what we have found concerning the LMM and SMM. Spindle cells are, however not infrequent among the NMM in our series.

There is a weak tendency of spindle cells to show slight atypia. In fact, no spindle cells with marked atypia were found. This may explain the relatively good prognosis of our patients with spindle celled tumours (Fig. 6). Most other authors did not find any significant relationship between cell type and prognosis (for further references see Krawson *et al.* 1971 Gartmann & Tritsch 1972, Little 1972). Our findings are, however in agreement with those concerning melanomas of the choroid and ciliary body which show the same cell types as the cutaneous MM (Calender *et al.* 1942).

The findings concerning cell type is difficult to interpret. They may explain partly why patients with LMM have a better prognosis than those with the two other types of MM but not why patients with NMM have a shorter survival than those with SMM.

Pigmentation

It soon became evident that this factor was difficult to grade. Most of the tumours showed a very uneven distribution of pigment, sometimes together with mixed cellularity as mentioned earlier. This phenomenon has already been described by other investigators (Thormann *et al.* 1970 Clark *et al.* 1975).

It is difficult to compare the relative frequency of our different grades of pigmentation (Fig. 7) with that of other investigations (Hardmeier *et al.* 1968 Cochran 1969 and Beardmore *et al.* 1970) because the grading methods are not the same. We have tried to extract two groups of MM (grade 0 and ++), which were as homogeneous as possible, the one including cases in which apparently all tumour cells produce melanin and the other amelanotic. We thereby hoped to make it reasonable to compare the survival of these two groups. We are aware that the pigmentation perhaps is not homogeneous throughout the whole tumour in some cases because we have only got a limited number of sections from each case.

Many investigators have failed to demonstrate any correlation between the pigmentation of MM and survival (for further references see Hino 1974 Chung *et al.* 1975 Elias *et al.* 1977). This may be because they have not compared homogeneous groups. However the histological findings of Hardmeier *et al.* (1968) and Little (1972) and the clinical findings of Sykes (1949) correspond with our results which show a better prognosis for the more pigmented tumours (Fig. 7). Furthermore, our findings fit in well with the significant tendency for amelanotic tumour cells in our series to show marked atypia and/or be rich in mitoses and/or to form NMM while moderate pigmentation seems to be associated with less atypia and/or fewer mitoses and/or LMM (Table 1). Our finding that the grade of pigmentation varies inversely with the mitotic count is in agreement with the findings of Gray & Pierce (1964) in transplanted MM cell-lines and with those of Schachtschabel (1971) and Kreider *et al.* (1975) in MM cell-cultures.

Provided that the grade of pigmentation indicates the differentiation of the tumour cell (Beardmore *et al.* 1970), the highly significant pattern in the relation between pigmentation and tumour type may partly explain the difference in the prognosis of the patients with various tumour types.

Atypia

The grade of atypia may vary from one area to another in a single tumour (Fig. 1) as mentioned earlier. We used the most atypical cells for grading, even when they were present only in a small part of

the tumour. In a few cases such small areas may be lacking when only one or a few sections of the tumour is examined as we have done.

The large number of indeterminate cases (139) in our series makes the incidence of the 3 grades of atypia difficult to compare with that of McGovern (1970) who has used the same system of grading. Both studies show, however, a dominance of grade ++.

The poor survival of our patients with marked atypia (Fig. 8) is in agreement with other investigations (McGovern 1970, Gartmann & Tritsch 1972).

The pattern of survival of our cases and the significantly high coincidence between grade + of atypia and LMM and grade +++ of atypia and NMM (Table 1) may partly explain the different prognosis of these tumour types.

Mitotic Count

The number of mitoses which are seen in a histological section depends not only on the mitotic rate but also on the duration of each mitosis (Iversen & Evensen 1962). Therefore the varying number of mitoses in melanotic tumours, perhaps even in different areas of the same tumour, may not necessarily illustrate variations of cell proliferation.

It is, however, extremely rare to find mitoses in benign naevi (Lund & Stobbe 1949), although some few mitoses may be seen in the dermal component of naevi of children (Allen & Spitz 1953). Contrarily Little (1972) and McGovern (1972) found mitoses in almost all of their MM. Empirically therefore it seems to be permissible to interpret the histological finding of even few mitoses in an adult melanotic tumour and many mitoses in a melanotic tumour of a child as a sign of malignancy.

Also the number of mitotic figures varies often considerably from one area to another in a primary MM. This may be due to the co-existence of cell clones with different ability of proliferation as mentioned earlier or to a varying vascularization of the tumour from one area to another (Tannock 1968). We cannot exclude the possibility that in a few cases our limited number of sections from each tumour has left us with a lower mitotic count than that which the examination of several transsections would have done.

The relative frequency of our 3 grades of mitotic count (Fig. 9) may be compared with the results of Little (1972) although he used a somewhat different grading method. We both found that a low mitotic count predominates and that there are few cases with many mitoses.

The prognosis is best among our cases of MM with few mitoses (Fig. 10). This is in agreement with the findings in some investigations (Cochran

1969, Hardmeier *et al.* 1968 and Little 1972) while other investigations disagree with this (Gerler & Thormann 1968, Fitzpatrick *et al.* 1972, Huvus *et al.* 1974).

As mentioned earlier there is a highly significant trend in the relation of atypia to mitotic count. Further there is a highly significant relation of LMM to few mitoses and of NMM to many mitoses. Provided that the mitotic count indicates the cell proliferation, these relationships (Table 1) and the prognostic value of the mitotic count may partly explain the different prognosis of the patients with various tumour types.

CONCLUSIONS

1) The following histological features indicate a good prognosis: spindle celled tumours, marked pigmentation, slight atypia and low mitotic count. Features correlated with a bad prognosis are: epithelioid celled tumours, little pigmentation, marked atypia and high mitotic count.

2) The most common finding was mixed cellularity together with little pigment, moderate atypia and low mitotic count. Most of these tumours were superficial spreading malignant melanomas.

3) When a lentigo maligna melanoma diverged from this common picture it was usually in the benign direction. When a nodular malignant melanoma diverged from the common picture it was usually in the malignant direction. When a superficial spreading malignant melanoma diverged it might be in either direction.

4) The prognostic value of tumour cell type and pigmentation were lower than that of atypia and mitotic count. The present study does not, however, allow any comparison between the prognostic value of these tumour cell features and that of tumour type.

We are thankful to Professor O. H. Hersen and Dr. K. Magnus, Cancer Registry of Norway, for valuable help and advice.

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A RETROSPECTIVE HISTOLOGICAL STUDY OF 669 CASES OF PRIMARY CUTANEOUS MALIGNANT MELANOMA IN CLINICAL STAGE I

3 *The Relation between the Tumour Associated Lymphocyte Infiltration and Age and sex, Tumour Cell Type, Pigmentation, Cellular Atypia, Mitotic Count, Depth of Invasion, Ulceration, Tumour Type and Prognosis*

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Larsen, T. E. & Grude, T. H. A retrospective histological study of 669 cases of primary cutaneous malignant melanoma in clinical stage I. 3. The relation between the (tumour-associated) lymphocyte infiltration and sex and age, tumour cell type, pigmentation, cellular atypia, mitotic count, depth of invasion, ulceration, tumour type and prognosis. *Acta path. microbiol. scand. Sect. A*, 86: 523-530, 1978.

A selected series of 669 primary malignant melanomas of the skin, stage I, was studied. The series included 146 lentigo maligna melanomas, 259 superficial spreading malignant melanomas, 194 nodular malignant melanomas and 130 undifferentiable malignant melanomas. The adjacent lymphocyte infiltration was graded and its prognostic value and its relation to the sex and age of the patient, tumour cell type, pigmentation, cellular atypia, mitotic count, depth of dermal invasion, tumour type and ulceration was studied. There was no significant relationship between lymphocyte response and sex and age of the patient and the tumour cell type. There was a highly significant relationship between a dense lymphocyte infiltration and superficial tumour invasion as far as the papillary-reticular interface. In contrast to the weak response associated with deeper invasion. When only tumours with invasion of the papillary-reticular interface were considered, there was no significant relationship between lymphocyte infiltration and pigmentation, cellular atypia, mitotic count, tumour type and ulceration. At the same level of invasion there was no difference in prognosis in relation to the density of lymphocyte infiltration. Nodular malignant melanomas surrounded by a dense lymphocyte infiltration had a significantly worse prognosis than was associated with a smudged lymphocyte response against the two other types of melanomas.

Key words: Melanoma, lymphocyte infiltration, prognosis.

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received 10.11.78 Accepted 5.1.79

In 1907 Handley observed that the lymphocyte infiltration around cutaneous malignant melanomas (MM) was especially marked in areas of tumour regression. In 1916 Sano described the 'halo nerve' in which regression is accompanied by a heavy lymphocyte accumulation, usually associated with the complete destruction of the tumour. Later immunological investigations have shown that the

inflammatory reaction around primary MM probably is due to an immunological host response (Lewis 1974, Edelson *et al.* 1975). The relationship between a marked tumour-associated lymphocyte infiltration and a good prognosis has been indicated for several types of non-melanotic tumours (for references see Thompson 1973). This is also true concerning MM (for further references see Garmann & Tritic 1972, Thompson 1973, Hansen &

Sjohén B Malignant melanoma of the skin. Acta Radiol 32 33-59 1949

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Lymphocyte Infiltration: PMM

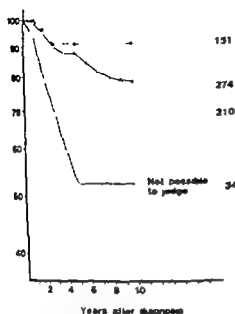


Fig 3 Survival curves illustrating the specific cumulative survival of the patients according to grade of lymphocyte infiltration around the tumour

Level of invasion

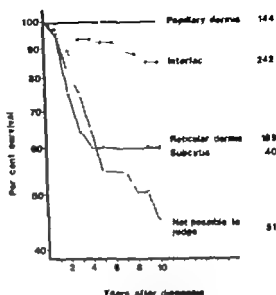


Fig 4 Survival curves illustrating the specific cumulative survival of the patients according to level of invasion growth of the tumour. The survival of 3 cases with intraepidermal tumour growth is not illustrated

TABLE 1 The Relationship between Lymphocyte Infiltration and Invasion, Tumour Cell Type, Atypia, Mitotic Count, Tumour Type, Depth of Invasion and Sex and Age. The Relationship between Tumour Type and Depth of Invasion

Factor compared	(N)	χ^2	d.f.	P	Significant trends among the determinate grades and types
Lymphocyte infiltration					
Depth of invasion	(18/47)	1.27	4	>0.1	0
Tumour cell type	(19/85)	8.27	9	>0.1	0
Atypia	(19/52)	8.50	9	>0.1	0
Sex and age ^a	(12/51)	11.03	9	>0.1	0
Mitotic count	(34/25)	16.80	9	>0.05	0
Papularisation	(26/76)	13.68	6	>0.02	0
Tumour type	(60/71)	25.93	9	>0.01	LMM ~ + + + + + NMM ~ + + +
Depth of invasion	(81/62)	57.65	10	<0.001	level II ~ + + + + + level IV ~ + + + + +
Tumour type compared with depth of invasion	(250/34)	709.41	12	<0.001	LMM ~ level II, NMM ~ level IV ~ V

^a The indeterministic grades and types eliminated

^b Degree of freedom

^c Men and women over and under 50 years, respectively

^d Lymphocyte infiltration

The complete tables are available from the author

McCarten 1974 and Kokoschka & Niebauer 1976). Some investigators, however, have not been able to confirm this relationship in MM (for further references see Beardmore *et al* 1970 Weldner 1973 Ellas *et al* 1977 and Lang *et al* 1977).

In the present paper we have examined the relationship of the lymphocyte response to depth of invasion, to the tumour type, to various histological features of the tumour cells (cell type, pigmentation, atypia and mitotic count) to ulceration of the tumour surface and to the sex and age of the patient. In addition, we have studied the prognostic value of this response in more detail than in a previous paper (Iversen *et al* 1975).

Little (1972) found a worse prognosis when plasma cells were present at the level of deepest invasion than when they were lacking. We have not considered the plasma cells.

METHODS

Together with O. H. Iversen we examined 669 cases of primary MM in clinical stage I which had been reported to the Cancer Registry of Norway from 1955-70 inclusive. The series includes 256 men and 413 women aged 6 to 93 years. The selection and the histological classification according to Clark's system (Clark 1967) have been reported earlier (Iversen *et al* 1975 Larsen &

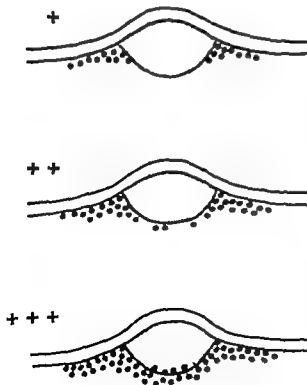


Fig 1 The grading of tumour associated lymphocyte infiltration used in this series.

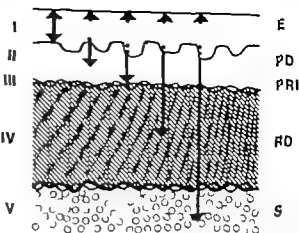


Fig 2 Levels of invasive growth according to Clark *et al* (1969).

Grude 1978a). The grading and prognostic value of tumour cell type, pigmentation, atypia and mitotic count and the relationship of these features to each other have been published in another previous paper (Larsen & Grude 1978b).

The amount of lymphocytes was graded + + + or + + + (Fig 1). The grade + + + meant an intense infiltration completely surrounding the invasive part of the tumour. Grade + indicated a slight infiltration which was limited to one or both sides of the invading tumour. Grade + + consisted of intermediate cases. Poor technical quality or complete disagreement between the three investigators resulted in the use of the term 'not possible to judge'.

Depth of invasion (Fig 2) was graded according to Clark (Clark *et al* 1969). Level I ~ epidermis, level II ~ papillary dermis, level III ~ papillary reticular interface, level IV ~ reticular dermis and level V ~ subcutis. If the lower part of the tumour was not represented the level was registered as 'not possible to judge'.

Ulceration of the tumour surface was registered as + (present), - (absent) or 'not possible to judge' (the surface not sufficiently represented).

We had no access to clinical information when the histological examination was done.

RESULTS

Fig 3 shows the relative distribution of the different grades of lymphocyte response. In 34 cases (5%) the lymphocyte infiltration was unclassifiable because the deeper part of the invasive tumour was not present.

Fig 3 shows also the specific cumulative survival rates (Cancer registry of Norway 1975) according to the lymphocyte response. The difference in survival between patients with grade + and grade + + + is significant at 5 years ($77.6 \pm 3.7\%$ versus 91.4

Two standard deviations.

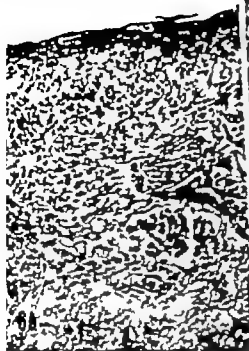


Fig. 6a A moderate lymphocyte infiltrate in the papillary dermis below lentigo maligna $\times 185$

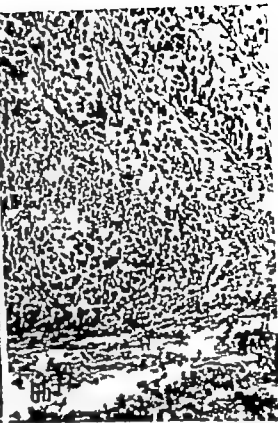


Fig. 6b A moderate lymphocyte infiltrate around and inside the lower part of a SIM at the papillary-reticular dermal junction which is depressed by the tumour $\times 135$

(the highly significant relationship between grade of lymphocyte response and level of invasion the relative incidence of the different grades in our series (Fig. 3) cannot be compared with that in other investigations because no information about this relationship has been given (e.g. Beardmore *et al.* 1970 and Thompson 1973).

A heavy lymphocyte infiltration is associated with a significantly better prognosis than a slight one in our series as long as the tumour type is not considered (Fig. 3). A heavy lymphocyte response does not however have any effect on the prognosis of SIM and only slightly on that of SSIM (Fig. 5). Koloschka & Nuberger (1976) found that the survival of patients with a slight and a marked lymphocyte infiltration around the tumour respectively was significantly different even at one level of tumour invasion. We have not been able to confirm this.

Before discussing the patterns of the various χ^2 -tests (Table 1) we must emphasize that the finding

of a significant pattern only means that the two particular features are not independent and not that they are directly dependent on each other. Two features may show a strong relationship because they are both connected with the same third feature. We cannot exclude the possibility that the present impressions will be changed when the result of the multiple regression analysis is available.

Relation to Depth of Invasion

It seems probable that the lymphocytes around the SIM represent an immunological host response as a barrier against the invasive growth of the tumour as proposed by Reed *et al.* (1975) (Fig. 6a + b). In this connection it is interesting to see how the lymphocyte infiltration decreases with increasing depth of invasion from the papillary dermis to the subcutis (Table 1). This is in agreement with what has been reported by Burg & Braun-Falco (1972) and by Hasebe *et al.* (1975). In fact, the pattern changes markedly when comparing tumours which

TABLE 2. *The Relationship between Lymphocyte Infiltration and Pigmentation, Cell Type Atypia Ulceration, Mitotic Count and Tumour Type at Level III of Dermal Invasion.*

Lymphocyte infiltration compared with	χ^2	d f	P	Obvious trends among the determinate grades and types
Pigmentation	9.33	6	>0.1	0
Cell type	2.42	4	>0.1	0
Atypia	7.30	6	>0.1	0
Ulceration	2.85	4	>0.1	0
Mitotic count	5.44	6	>0.1	0
Tumour type	9.99	6	>0.1	0

Degrees of freedom

The complete tables are available from the author

$\pm 2.9\%$) and at 10 years after diagnosis ($68.9 \pm 5\%$ versus $91.4 \pm 2.9\%$). This difference disappears when patients with LMM and NMM are considered alone, but is still present in the SNM groups at 5 years after diagnosis ($80.8 \pm 5.7\%$ versus $91.8 \pm 4.0\%$) and 10 years after diagnosis ($71.0 \pm 7.9\%$ versus $91.8 \pm 4.0\%$).

Fig. 4 shows the specific cumulative survival of patients having tumours with different level of invasion. The survival rates diminish markedly as the level of invasion deepens from the papillary reticular interface to the reticular dermis. The difference is significant after 5 years ($92.4 \pm 2.6\%$ versus $71.0 \pm 4.1\%$) as well as after 10 years ($84.3 \pm 4.0\%$ versus $60.3 \pm 5.4\%$). It is also significant in each particular type of tumour. According to Hermanek *et al.* (1976) the level of invasion is of greater prognostic importance than the tumour type. If we consider only the 242 cases of MM which invade the papillary reticular interface there are about the same number with grade + and grade +++ of lymphocyte response (64 resp. 65 cases). The prognosis of these 2 groups is not significantly different (81.8% versus 81.2% alive after 5 years and 66.4% versus 76.6% alive after 10 years).

Table 1 shows the relation of lymphocyte infiltration to sex and age and to the various histological criteria of MM mentioned above and in addition of tumour type to level of invasion according to χ^2 tests. Because of the strong linkage of some of the indeterminate grades and types to each other we have eliminated these features when evaluating the χ^2 value without reducing the degrees of freedom. The highly significant relation of lymphocyte infiltration to tumour type and level of invasion and between these

two features is due to obvious trends among the determinate grades and types.

Table 2 illustrates the relation of lymphocyte infiltration at level III of invasion to the other features mentioned above. No significant trends are found.

DISCUSSION

When grading the lymphocyte infiltration it was often difficult to draw the line between grade + and ++ and between ++ and +++ This resulted in a large group of MM with grade ++. Because

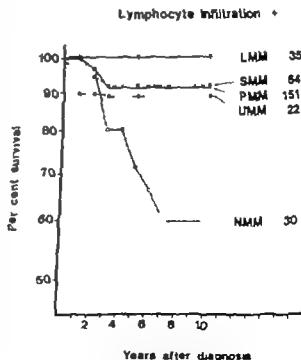


Fig. 5 Survival curves illustrating the specific cumulative survival of patients with grade +++ of lymphocyte infiltration around the tumour according to each tumour type.



Fig. 6a. A moderate lymphocyte infiltrate in the papillary dermis below a lentigo maligna $\times 185$.

Fig. 6b. A moderate lymphocyte infiltrate around and inside the lower part of a NM at the papillary-reticular interface which is depressed by the tumour $\times 135$.

of the highly significant relationship between grade of lymphocyte response and level of invasion the relative incidence of the different grades in our series (Fig. 3) cannot be compared with that in other investigations because no information about this relationship has been given (e.g. Beardmore *et al* 1970 and Thompson 1973).

A heavy lymphocyte infiltration is associated with a significantly better prognosis than a slight one in our series as long as the tumour type is not considered (Fig. 3). A heavy lymphocyte response does not, however, have any effect on the prognosis of NM and only slightly on that of SM (Fig. 5). Koleschke & Vachauer (1976) found that the survival of patients with a slight and a marked lymphocyte infiltration around the tumour respectively was significantly different even at one level of tumour invasion. We have not been able to confirm this.

Before discussing the patterns of the various χ^2 -tests (Table 1) we must emphasize that the finding

of a significant pattern only means that the two particular features are not independent and *not* that they are directly dependent on each other. Two features may show a strong relationship because they are both connected with the same third feature. We cannot exclude the possibility that the present impressions will be changed when the result of the multiple regression analysis is available.

Relation to Depth of Invasion

It seems probable that the lymphocytes around the NM represent an immunological host response as a barrier against the invasive growth of the tumour as proposed by Reed *et al* (1975) (Fig. 6a + b). In this connection it is interesting to see how the lymphocyte infiltration decreases with increasing depth of invasion from the papillary dermis to the subcutis (Table 1). This is in agreement with what has been reported by Burg & Braun-Falco (1972) and by Hanebo *et al* (1975). In fact, the pattern changes markedly when comparing tumours which

invade the papillary-reticular interface with those invading the reticular dermis. This may correspond to the significant difference in survival of patients with tumours infiltrating level III and IV (Fig. 4).

Our observations may indicate that a MM produces a weaker immune response when it invades the reticular dermis than at more superficial levels. We have, however, no means of knowing whether a highly malignant MM which breaks through a lymphocyte barrier subsequently causes this to decrease, or whether it is the lymphocyte barrier which for some reason primarily diminishes and gives the tumour cells free access to further invasion (Clark 1967).

A sparse lymphocyte infiltrate does not necessarily mean a low immunogenicity of the tumour or a low immunocompetence of the patient. It might as well be due to blocking serum factors which seem to be circulating antigen antibody complexes (Sjögren *et al* 1971). Blocking serum factors are found especially in patients with metastatic MM (Heppner *et al* 1973). This is in accordance with the fact that a lymphocyte infiltrate is seldom found around metastatic MM (Payan *et al* 1970, Burg & Braun-Falco 1972). The reason for the decrease of the lymphocyte reaction with growing depth of invasion might be the production of such blocking serum factors, and it might thereby indicate that the tumour has metastasised. Lewis (1974) suggests another possibility that the MM cells express different antigens at different stages of the disease.

Relation to Tumour Type

The highly significant trend in the relation of lymphocyte infiltration to the various tumour types (Table 1) probably depends upon their different ability of invasive growth. The significance disappears in the 242 cases showing invasion level III (Table 2) although there is still a weak tendency of LMM to show grade ++ or +++ of lymphocyte infiltration and of NMM to show grade +.

Relation to Histological Criteria of the Tumour Cell

According to Clark *et al* (1975) so-called «intralesional transformation» may give rise to collections of tumour cells which originate from different cell clones and lead to different lymphocyte infiltrations. This might be because the immunologic response is dependent upon tumour cell type as well as on grade of pigmentation, atypia and number of mitoses.

Like Gartmann & Fritsch (1972) we found no significant relationship between the lymphocyte infiltration and cell type or atypia (Table 1 and 2).

The lack of any significant relationship between lymphocyte response and pigmentation (Table 1 and

2) is in agreement with the findings of Morton *et al* (1968). They suggested that melanin has no immunogenicity.

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Relation to Ulceration

We found no significant pattern in the relationship of lymphocyte reaction to tumour ulceration (Table 1 and 2). This is in agreement with the findings of Beardmore *et al* 1970. It is our impression that ulceration of the tumour surface has as its first effect an increase in the number of neutrophils in an inflammatory infiltrate. Little (1972) has noted the presence of many plasma cells when a MM is ulcerated, but we have no information on this point.

Relation to Sex and Age

As shown in Table 1 we have found no trend concerning the relation of lymphocyte response to sex and age of the patient. The bad prognosis in older men found by Magnus (1977) is therefore not reflected in the tumour-associated lymphocyte infiltration in this series.

CONCLUSIONS

- 1) We have not been able to demonstrate any significant relationship between lymphocyte infiltration and tumour cell type, pigmentation, atypia, mitotic count, and sex and age of the patient. Neither was there any significant relation of lymphocyte infiltration to tumour type at one level of invasion (level III).
- 2) A weak lymphocyte response was found especially when the tumour invaded the deeper part of the dermis or the subcutis where the prognosis was inevitably poor. The lymphocyte infiltration was especially marked when the MM invaded the upper part of the dermis (level II). It did not, however, make the prognosis of the patients with a nodular malignant melanoma as good as that of the patients with the two other tumour types. Further there was no difference in the prognosis of cases with a dense lymphocyte response and those with a weak one when the tumour had reached invasion level III. The prognostic meaning of a dense lymphocyte infiltration seems thus to be limited.
- 3) The obvious shift at the papillary-reticular interface to a sparse lymphocyte response together with the significant worsening of the prognosis when the level of invasion is increased from III to

indexes that the papillary-reticular interface is a topologically very important area.

We wish to thank Professor O H Hensen and Dr K Magnus for valuable help and advice.

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IMMUNOFLUORESCENT MICROSCOPY FINDINGS IN MINIMAL OR NO CHANGE DISEASE AND SLIGHT GENERALISED MESANGIOPROLIFERATIVE GLOMERULONEPHRITIS

Fluorescent Microscopy Results Correlated to Symptoms and Clinical Course

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Abstract. 5 Immunofluorescent microscopy findings in minimal or no change-disease and slight generalised mesangioproliferative glomerulonephritis. Fluorescent microscopy results correlated to symptoms and clinical course. *Acta path. microbiol. scand. Sect. A, 86 531-542, 1978.*

Seventy-two kidney biopsies from patients with clinical evidence of glomerulonephritis (GN) were examined by immunofluorescent microscopy (IFM) using 1 μ m cryostat sections and optimal excitation interference filters. The biopsies were classified at light microscopy (LM) as normal, minimal lesion, or mild generalised proliferative GN. As a spot check on the LM diagnosis 19 biopsies were examined by electron microscopy (EM). Comparison of IFM findings and the clinical symptoms and clinical course of the disease showed:

- 1 that in patients with deposits of IgG-IgA/C₃, haematuria and systemic disease were significantly more frequent, and there was less tendency to complete remission, than in patients with other deposits
- 2 that IgG-IgA nephropathy is probably brought by C₃ activation through the alternative pathway
- 3 that all biopsies with deposits of IgA/C₃ were from patients with a clinical history of previous, recurring upper respiratory tract infection
- 4 that 18 out of 19 biopsies where no deposits could be demonstrated, were from patients with no clinical history or clinical evidence of infection

Apart from this no significant correlation was found between the fluorescent findings and clinical symptoms in GN or its clinical course. No specific IFM findings could be related to any of the LM changes. IFM results therefore suggest that the presence of some immunoglobulins may be interpreted as showing involvement of immune mechanisms only at a purely secondary level. However this does not exclude the possibility that immunoglobulin deposits can be of importance for the development of a protracted or perhaps chronic course in the disease, and therefore the importance of deposits should first be assessed after a longer period of years than in the present work.

Key words: immunofluorescent microscopy, kidney disease, glomerulonephritis

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Method

Immediately after the biopsy was obtained, each specimen was divided under a stereomicroscope for LM and IFM, and also for EM if sufficient tissue was available.

Fluorescent Microscopy

IFM studies were carried out without knowledge of clinical data or the result of LM investigations. Tissue for IFM was frozen using dry ice and blocked in Tissue-Tek®-pembone (Ames Laboratory) before 1 µm sections were cut at -4°C on a Leitz ultracytome® with an automatic section remover. To achieve suitability thin sections only new or newly sharpened knives were used from Karl Heitz Dorsch (Hedelfberg).

The sections were mounted on clean slides and treated by a direct fluorescent technique as shown in Table 1.

The remaining tissue was stored at -79°C.

Antisera

Specific antisera react to human immunoglobulins IgG, IgM, IgA as well as complement C₃ and fibrin/fibrinogen are used in all studies. After January 1974 the studies were supplemented by testing for IgD, IgE, C₄, C₅ as well as properdin. Some antisera were obtained conjugated with fluorescein-isothiocyanate (FITC) from commercial laboratories: Meloy Laboratories, Biological Products Division, Behringwerke AG, Dakopatts A/S, Dakoimmunoglobulins A/S, Hyland Laboratories, INC as well as Nordic Immunological Laboratories. Other antisera were obtained unconjugated from the same firms and tested for species-specific reaction by double immunodiffusion (Ouchterlony technique) (Klauser 1969) before conjugating with FITC and purification in a column with Sephadex G 25 medium. Rabbit anti-human properdin was kindly provided by Professor Anne Bruus Larsen, Microbiological Institute, University of Lund, Sweden.

The conjugated antisera were used in dilutions of 1:5–1:40 and in the case of fibrin/fibrinogen 1:300. As a control for specific fluorescence of C₃, tissue sections were heated to 56°C for half an hour. As a control for all surfaces a blocking technique was used by treating the tissue sections with unconjugated antisera before application of the conjugated sera (Table 1).

Immunofluorescent microscopy was performed using a Zeiss non-critical fluorescence microscope with transmit and light and Dark Field Ultravioletfilter N A 1.2/1.4 and objective 40:0.75 to 1.0 N A. Oil with immersion 10:1 µl. Light source HBO 700 W/4 pressure mercury lamp. Filter Primary interference filter cut off 440 nm and cut on 500 nm, with accompanying transmission of narrow red band of about 3–5 nm at some point between 640 nm–660 nm.

Secondary 1 µm glass filter matched with the primary filter (R. J. and O'Brien 1971).

Light Microscopy (Dr. Claus Bruus)

The tissue was fixed in 4% formaldehyde buffered to pH 7.6 (Lillie, Duval), embedded in Paraplast® cut into 4, 3 and 2 µm sections and stained with H & E, PAS

+ H. picroic acid + sirius red, Masson's trichrome and silver methenamine (Jones 1951) + H & E. Microscope: Leitz ortholux.

Electron Microscopy (Dr. Finn Jørgensen)

The tissue was fixed in 3% glutaraldehyde at pH 7.4 followed by dehydration in increasing concentrations of alcohol after fixation in 1% osmium tetroxide, and embedding in Epon. Ultra-thin sections were cut on an LKB I ultramicrotome and examined on a Philips 100 electron microscope.

Statistical Methods

The Chi square test was used to test for any correlation between the fluorescent findings and the clinical symptoms and clinical course of the disease.

RESULTS

Age and sex distribution appear in Fig 1.

The length of time from the onset of kidney disease to the first kidney biopsy does not differ significantly in the 3 groups, (2 weeks–15 years). Prior to the time treated with corticosteroids, and a total of 4 patients were receiving cytostatic treatment. One week after the date of biopsy 40% of the patients were on corticosteroid and/or cytostatic treatment. Elevated blood pressure was found in 13% of patients with minimal lesion, in 15% of patients with normal glomeruli, and in 18% of patients with proliferative GN.

Fluorescent Microscopy Findings

are shown in Table 2, and the terminology used is seen in Table 3.

In 27% of the biopsies no deposits were found whilst deposits were demonstrated in 73%.

Distribution of Deposits

The average of glomeruli examined by IFM was 5 per biopsy (range 2–17). The deposits were generalised, apart from one case where they were focal.

Pattern

Granular deposits were found in all biopsies. Two sections, 1 µm and 4 µm in thickness, were examined from each biopsy. The deposits were more finely granular and it was possible to identify the localisation of the deposits more precisely in the 1 µm sections. Interrupted linear deposits were demonstrated in the capillary loops in 13 of the biopsies.

Localisation

Segmental deposits were demonstrated in 5 biopsies (1 normal, 4 with slight proliferative GN). In the remaining biopsies the deposits were global.

Fresh kidney tissue obtained by percutaneous needle biopsy for examination by light microscopy (LM) from patients with clinical evidence of kidney disease, (Iversen & Bruun 1951), first made it possible to compare morphological changes with the actual clinical picture. A pre-requisite for using the results by this method was that reliable criteria were available for the individual microscopist when evaluating the morphological changes. Such criteria for morphological LM classification of the glomerulopathies were published by Pirani and Salinas Madrigal (1968) and by Habib (1970) which made it possible to compare material in different studies. In spite of uniform characteristic and well-defined LM changes there is no good correlation between these and the clinical picture, (Cameron 1975) and using conventional LM discrimination between normal glomeruli and very slight proliferative changes is fraught with uncertainty. An extended light microscopic method using nuclear counting of the individual cell fractions making up the glomerulus is perhaps a more promising method for separating these groups (Kawano *et al* 1971 Hanberg Sørensen 1972 Bohle *et al* 1974). However in some instances the morphological changes are so slight that classification is only possible if EM is also used. At the present time immune mechanisms are generally accepted as pathogenetic in several forms of GN and IFM techniques are increasingly used as an additional aid to morphological studies to demonstrate immunoglobulin and complement fractions as well as fibrin bound in the glomerulus (Wilson and Dixon 1974a).

In the present study performed from 1972–1976 at Kommunehospitalet, Copenhagen in the Clinical Chemistry Department, the clinical findings were correlated to the IFM studies in 72 kidney biopsies which on conventional LM were classified as normal minimal lesion or slight mesangioliproliferative GN.

MATERIAL AND METHODS

Selection of Patients

Patients included in this study were required to have had clinical evidence of GN with at least one of the following symptoms, and were observed for at least 3 months.

Haematuria: urine with at least 2 erythrocytes/high-power field (Larcom and Carter (1948). Proteinuria > 0.5 g/day (Reiman and Levinsky 1971). On LM the biopsies were required to have been classified as 'normal', or minimal lesion (minimal or no change-disease) where glomeruli were characterised by no or slight increase in mesangial width without evidence of cellular

proliferation. Slight generalised mesangioliproliferative GN was characterised by mesangial and possibly endothelial cell hypercellularity.

LM examination of each biopsy was based on at least 13 glomeruli. The IFM studies were based on at least 2 glomeruli.

There were 72 biopsies fulfilling the above conditions. Of these 2 were excluded from the study when EM revealed epimembranous GN and dense deposit disease (Habib *et al* 1975). A minimum of 13 glomeruli for assessment by LM (Thomson 1965) was chosen to reduce the possibility of missing focal GN and especially focal segmental sclerosis with juxta medullary localisation.

In 23 cases there was sufficient tissue for electron microscopy (EM) examination, and 19 of those contained glomeruli. The results of EM studies on these 19 biopsies were used as a spot check on the LM diagnosis.

The term nephrotic syndrome was used when serum albumin ≤ 20 g/l (Screiner 1971), with proteinuria of > 5 g/day (Robson 1967).

Infection. Clinical history of upper respiratory tract infection shortly before the presentation of the kidney disease, and/or an antistreptolysin titre (ASO) > 600. Elevated blood pressure was defined as diastolic ≥ 100 mm Hg.

Reduced kidney function: serum creatinine > 1.5 mg% or creatinine or ^{51}Cr EDTA clearance < 1 ml/sec.

TABLE 1 Preparation of Specimens for Immunofluorescent Microscopy

1	Dried in warm air for 1 hour
2	Phosphate buffered saline (PBS) pH 7.1 $\times 5$ min
3	Fixed in 93% (w/w) ethanol
4	Phosphate-buffered saline (PBS) pH 7.1 $\times 5$ min
5	Dist. water $\times 5$ min
	a. Fluorescein-labelled antibodies
6	Application.
	b. Unlabelled antibodies (blocking)
6a	
1	Moist chamber 30 min
2	Running tap water 1 min
3	Dist. water 2×5 min
4	PBS pH 7.1 2×5 min
5	Dist. water 2×5 min
6	Coverslip with glycerol trebuffered pH 8.4
6b	
1	Over night in moist chamber
2	Dist. water 2×5 min
3	PBS pH 7.1 2×5 min
4	Dist. water 2×5 min
5	After that 6a 1–6

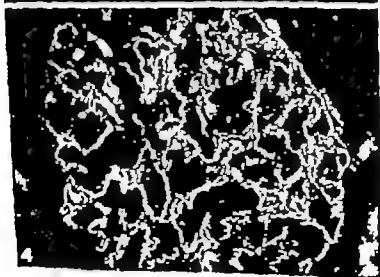
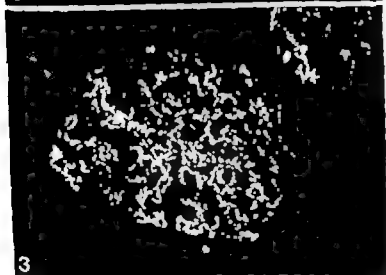
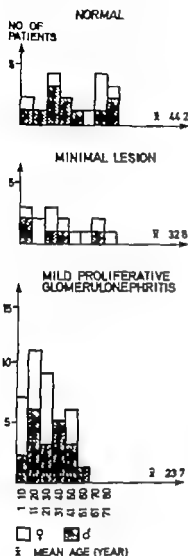


FIG. 1
SEX, AGE AND MEAN AGE



In 8% the deposits were only localised to the mesangium (Fig. 2)

In 76% of the biopsies the deposits were found equally distributed to the mesangium and basement membranes of the capillary loops (Fig. 3).

In 16% the deposits were mainly localised to the basement membrane of the capillary loops and less markedly to the mesangium (Fig. 4)

No localisation was specific for any of the 3 light microscopy groups and no immunoglobulin class or C_3 was found to correlate significantly to any specific localisation.

Bowman's Capsules

Deposits were found in 9 biopsies and in all of them the distribution was focal.

In 6 there were linear interrupted deposits of either IgG, IgA or C_3 localised to the basement membrane itself. In 3 cases the deposits were

granular and localised on the outside of the basement membrane of Bowman's capsule. Immunoglobulin and complement were not demonstrated together. No deposits were found in the epithelium of Bowman's capsule.

Deposits

No combinations of immunoglobulin (with or without C_3) could be related specifically to any of the LM groups. All biopsies were investigated for glomerular deposits of IgG, IgM, IgA and C_3 . The findings are shown in Table 2 and Table 4.

As only some biopsies were examined for IgD, IgE, C_4 and C_5 these are not shown in the tables, but the results were as follows.

IgD 37 biopsies were examined but deposits were demonstrated in only 1 patient (myelomatous, classified as a myeloma of IgM-type).

IgE 42 biopsies were tested, none was positive.

C_4 of 22 biopsies examined only 2 showed deposits and these were found with IgG/ C_3 and C_4 .

C_3 36 biopsies were tested. Granular and linear deposits were demonstrated in 10 cases and in 3 of these this was the only finding on the biopsy.

Properdin Findings are shown in Table 4 and were demonstrated in only 2 cases where the deposit was granular and found in the mesangium along with IgA/ C_3 .

Fibrinogen/fibrin were shown to be present in 18 biopsies as seen in Table 4. The deposits were granular to linear and were chiefly localised in the basement membrane of the capillary loops.

In 4 cases the deposits were localised solely to the mesangium.

Results of the Electron Microscopy Studies

Electron microscopy examination of 19 biopsies (indicated in Table 2) showed that 1 biopsy which was classified as minimal lesion on LM was in fact proliferative GN (459B) with hyperplasia of the mesangial cells and an increase of intercapillary substance. No «humps» were demonstrated, but

Fig. 2 ($\times 325$).

Granular deposits of IgA in the Glomeruli localised solely to the mesangium.

Fig. 3 ($\times 250$).

Glomerular deposits of granular complement C_3 equally distributed to the mesangium and basement membranes of the capillary loops.

Fig. 4 ($\times 520$).

Glomerular deposits of granular IgG mainly localised to the basement membranes of the capillary loops and less markedly to the mesangium.

TABLE 4. Pertinent Data on the Patients Studied

[illegible]

TABLE 4 Deposits and Their Localisation in the Glomerulus Correlated to Clinical Information

	No pts	Haematuria	Proteinuria	Nephrotic syndrome	BP elevated	GFR decreased	Leucocytosis	Systolic deviate	Complete remission	> 12 months	< 12 months of disease before biopsy (months)	Fibrin	Properdin stained	
Deposits														
lgG, MAC + C ₃	4	25	100	50	0	25	25	75	75	14	25	75	0	0/1
lgG, A + C ₃	10	80**	90	20	20	20	40	60	10**	9	30	70	60	0/6
lgA + C ₃	5	40	60	0	0	0	100	40	40	9	60	40	70	2/4
lgM + C ₃	6	17	100	33	17	0	33	0	50	9	0	100	33	0/0
lgG, M + C ₃	8	13	100	38	25	0	25	15	38	10	25	75	25	0/0
lgG, C ₃	5	20	40	0	20	0	0	20	40	5	20	80	20	0/1
Other Groups + C ₃	7	71	86	14	14	29	43	14	28	10	14	86	1	0/1
C ₃	7	71	86	14	14	29	43	14	28	10	14	86	1	0/1
lgG, MAC, A, M, C ₃	6	50	100	33	33	67	50	33	30	6	17	83	17	0/4
No Deposits	19	47	100	32	21	11	5**	16	32	13	16	84	11	0/5
Total	70	44	90	26	19	16	30	29	37	10.5	21	79	26	2/22
Localisation														
Yes	4	75	100	0	0	0	100	25	25	8.5	25	75		
Yes Cap	38	38	87	25	13	25	25	13	25	12.6	25	75		
Cap	9	41	85	26	21	18	36	36	33	8.4	23	77		
Total	51	43	88	24	18	18	39	31	33	9.8	24	76		
χ^2 test obs period p<0.05 p<0.001														

plex-nephritis) were demonstrated in 73% of the patients, whilst these could not be demonstrated in 27%. Linear nephritis could not be demonstrated. Linear nephritis, which is considered to be caused by circulating autologous antibodies directed against the glomerular basement membrane (Lerner *et al* 1967), is demonstrated most often as extracapillary GN where it can present solely as kidney disease (Jöres *et al* 1974) or as part of Goodpasture's syndrome (Wachlock *et al* 1969). But in few instances it has also been demonstrated in generalised proliferative GN (Moré manager 1972), and in minimal LM findings (Wardle *et al* 1975).

As linear nephritis is found in less than 2% of human GN and extracapillary GN was not included in the present study, it is not at all surprising that linear nephritis was not found in the 70 kidney biopsies investigated here.

Nineteen biopsies (27%) showed no glomerular deposits and this group differed significantly ($p < 0.001$) from patients with glomerular deposits in having a normal ASO and no history of infection prior to biopsy. These patients without glomerular deposits were distributed more or less equally in the

3 light microscopy classes. Clinical and prognostically they did not differ from the group of patients with glomerular deposits. They thus cannot be accepted as forming a group of patients with 'minor changes'. The aetiology and pathogenesis of the glomerulopathy and symptoms in these patients therefore remained unclarified. It is not possible to exclude that some of the patients may primarily have had an immune complex type of glomerulopathy where the glomerular-bound immunoglobulin and C₃ had disappeared by the time of biopsy. However, other studies on re-biopsied patients have shown deposits up to several years after the presentation of GN even without activity of the disease (Teiser *et al* 1969). Other immunopathogenic mechanisms, such as cellular hypersensitivity (Rouf 1973), cannot be excluded as a cause of GN in patients without deposits of immunoglobulin. Such a hypothesis has been proposed by Bendixen (1968) and Elvers (1976). In addition organic solvents have come to the forefront as a possible contributory factor in kidney disease, including glomerulopathy (Zimmerman 1975; Ransilov *et al* 1977).

It is possible that glomerular deposits could not

TABLE 3 *Terminology and Definition*

Terminology	Abbreviation	Definition
Fluorescence		
Positive = (+) + (+ +)	+	Bright fluorescence, weak, moderate and heavy
Negative =	-	Complete absence of fluorescence
Localisation:		
Mesangial	(Mes)	Glomerular intercapillary region
Capillary basement membrane	(Cap)	Within/along the capillary basement membrane
Bowman's capsule	(Bow)	In Bowman's capsule
Distribution:		
Generalised	(G)	All glomeruli in specimen
Focal	(F)	One/some glomeruli in specimen
Global	(Glo)	Including all portions of the individual glomerulus
Segmental	(S)	Including only parts of the individual glomerulus
Pattern		
Granular	(Gr)	Fine/coarse granular fluorescence
Linear	(L)	Linear continuous or discontinuous fluorescence within/along the basement membrane

electron-dense deposits were seen between the basement membrane and the intercapillary cells in a few lobules

In one biopsy which on LM was classified as generalised proliferative GN (603), it was only possible to demonstrate minimal lesion on EM characterised by increased intercapillary substance with increase in mesangial width. 5 biopsies, taken from patients with no evidence of systemic disease (508 511 405 585 603) were on EM classified as minimal lesion. In 4 of these glomerular deposits of immunoglobulin and/or C_3 were demonstrated by IFM but EM examination did not show electron dense deposits in spite of there being at least 2 glomeruli available for study

IFM Findings Correlated to the Clinical Observations

In Table 4 the IFM findings in the renal corpuscle are correlated to the clinical observations. Where deposits of IgG IgA/ C_3 were found there was a higher frequency of haematuria and kidney disease secondary to other primary disorders, (3 Henoch-Schönlein purpura, 1 scleroderma, 1 Crohn's disease, and 1 possible myositis). At the same time there was less tendency to complete remission. Patients with deposits of IgA/ C_3 had all had recurring infection in the upper respiratory tract for several years as well as shortly before the kidney condition presented. In addition the ASO was > 600 in all of them at the time of kidney biopsy 18 out of 19 patients with no deposits had no clinical history of previous infection and the ASO was normal. In 5 patients without systemic disease similar symptoms and clinical course were seen with mesangial deposits of IgA/ C_3 in 2 and IgG-IgA/ C_3 in 3 patients. They had intermittent microscopic haematuria which was periodically macroscopic (then always associated with recurrence of upper respiratory tract infection), and all had only negligible proteinuria.

DISCUSSION

Two different immunopathogenic mechanisms (Dixon 1968), are considered responsible for about 85% of the cases of human GN (Wilson and Dixon 1974b). The one mechanism, which accounts for less than 5% according to Merial (1974), is characterised by a continuous linear fluorescent pattern (linear nephritis) caused by linear deposited immunoglobulin and C_3 in the basement membrane of the capillary loops (Lerner *et al* 1967 Poskitt 1970). This pattern was found in 14 of our 1700 biopsies (1.2%).

The other mechanism typically has a fluorescent pattern characterised by granular deposits of immunoglobulin and C_3 in the glomeruli.

Our knowledge and concepts of the importance of these immunopathogenic mechanisms in GN are based especially on experimental animal studies by among others, Germuth and Pollack (1958) McCluskey *et al* (1960) Dixon *et al* (1961) and Cochrane (1969) where GN was induced using well defined antigens e.g. Bovine albumin or antigen-antibody complex. This is contrast to human GN where the antigen has only been demonstrated with certainty in a few instances (Koffler *et al* 1967). It is therefore not justified to refer to deposits of immunoglobulins and C_3 as immune complex deposits on IFM examination. In the present study granular deposits of immunoglobulin/ C_3 (wcom

demonstrated, the symptoms and the course of the disease seem therefore to exclude the possibility of a strict, causally applicable IFM classification where IM shows normal glomeruli with minimal lesions and slight generalised proliferative GN.

It is therefore a pertinent question whether the presence of immunoglobulins, other than IgA and IgG-IgA, shows anything more than involvement of immune mechanisms at a purely secondary level, without, however discounting that their presence can be a contributory factor in the development of damage in the disease (Ulanowicz and Dixon 1965) or an indicator of a chronic course.

Perhaps, therefore, the IFM findings should first be correlated to the clinical picture after several years instead of the shorter observation period in this study.

I gratefully acknowledge the financial support of The Swedish Medical Research Council (1 yr \$12 2669) and my Christian X Foundation. My thanks are due to Mrs Andersson, Karin Sellberg and Birgit Høy for skilful technical assistance.

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be demonstrated in these patients on IFM examination because of poor filter properties (Markham *et al* 1973).

This can be excluded because all investigations were performed using interference filters (Rygaard and Olsen 1971) with optimal FITC transmission (Tomlinson 1973). The conditions must therefore be considered optimal for IFM investigations.

On the other hand the use of an indirect fluorescent technique could possibly have revealed a greater number of IFM positive findings in the biopsies (Nairn 1969; Tarantino *et al* 1973).

In 51 (73%) biopsies glomerular deposits of immunoglobulin and/or C_3 were found. The individual groups of patients with deposits localised either solely to the capillary loops solely to the mesangium or localised simultaneously to the capillary loops and the mesangium showed no significant correlation of the glomerulopathy to infection, other clinical symptoms or the course of the disease. Patients with deposits solely localised to the capillary loops did not form a well-defined group with a uniform clinical picture or prognosis. This might have been expected from a theoretical point of view based on traditional concepts of «complex nephritis» which is characteristic of experimental models (experimental serum sickness) studied among others by Kniker and Cochran (1965) and Germuth *et al* (1967). Only a little is known about factors which control the precise localisation of immune complexes within the glomerulus, (McCluskey 1974). However it is not possible to exclude that some of the patients with deposits localised simultaneously to the capillary loops and mesangium may primarily have had deposits which were solely localised to the capillary loops but which later shifted to the mesangium as a result of steroid treatment (Germuth *et al* 1968).

Localisation of deposits solely to the mesangium showed no correlation to the clinical symptoms or course of the disease, but on the other hand in these patients there was a preponderance of deposited IgA and the combination IgG-IgA. Of the classes of immunoglobulins deposited (IgG, IgA, IgM and their combinations) only IgA/ C_3 and IgG-IgA/ C_3 were found to correlate significantly to individual clinical symptoms and the clinical course.

IgA/ C_3 is rarely seen and nothing is known about the mediator mechanisms which accompany IgA deposition in glomeruli (Hjman *et al* 1973). However streptococci, as shown in the present study can be a contributory aetiological factor in these patients' nephropathy.

In 2 of our cases (without systemic disease) with a clinical picture of IgG-IgA nephropathy (Berger and Hinglais 1968; Berger *et al* 1969), we found

the biopsy was obtained less than 2 weeks after presentation of the kidney disorder and IgA/ C_3 was deposited in the mesangium along with properdin, but without C_{1q} , so that activation of C_3 must have occurred via the alternative pathway (Fearon *et al* 1974). But whether this involves an immunopathological reaction is not yet clear (Muller Eberhard 1974). In 3 other patients (without systemic disease) with mesangial deposits of IgG-IgA/ C_3 but without demonstrable properdin, the same symptoms and course were seen. The biopsy in these cases was performed more than 3 months after the kidney disease presented. Therefore it is not possible to exclude that the so-called IgG-IgA nephropathy is caused in its early phase by IgA activation of C_3 via the alternative pathway and that there is then a later secondary C_3 activation by complexes containing IgG which may lead to protracted and perhaps chronic course of the disease. However the LM changes seem to be of importance for the prognosis in this category of patients (Levy *et al* 1973), and the disease apparently has a more protracted and chronic course than was previously supposed by Berger *et al* (1969), both in children (McEneaney *et al* 1972) as well as in adults (van der Peet *et al* 1977).

Three patients with Henoch Schonleins purpura also had deposits of IgG-IgA/ C_3 solely localised to the mesangium and it was not possible by IFM to separate these patients from the patients with «IgG-IgA/ C_3 -nephropathy» without taking the clinical findings into account.

Black *et al* (1970) have pointed out that precise morphological separation of normal glomeruli and glomeruli with slight proliferative GN is impossible using conventional LM. This may to some degree be the reason for lack of correlation between the IFM findings and the morphological changes used as a basis for the LM classification in the present study.

Such an explanation was supported by the EM spot checks in the present study where 11% (2/17) of the biopsies were mistakenly classified in the LM groups. The same errors in classification may also apply to the remaining biopsies which were not examined by EM.

Deposits of immunoglobulin and/or C_3 in the mesangium and along the basement membrane of the capillary loops were demonstrated in 6 biopsies (2 normal, 4 minimal lesion) in patients without systemic disease, where the LM diagnosis was confirmed by EM.

It was therefore not possible to confirm a clear relationship between negative IFM findings and the glomeruli which on LM were normal or showed «minimal lesions», as stated by Morel Maroger *et al*. (1972) and Habib and Kleinkecht (1971).

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The lack of correlation between the deposits

IMMUNE DEPOSITS IN GENERALISED MESANGIOPROLIFERATIVE GLOMERULONEPHRITIS

Fluorescent Microscopy Findings Correlated to Symptoms, Clinical Course and Immunosuppressive Therapy

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Abstract. Immune deposits in generalised mesangioproliferative glomerulonephritis. Fluorescent microscopy findings correlated to symptoms, clinical course and immunosuppressive therapy. *Acta path. microbiol. scand. Sect. A, 86 543-552, 1978.*

One hundred and fourteen kidney biopsies from 106 patients with clinical evidence of glomerulonephritis (GN), and a diagnosis in light microscopy (LM) of generalised mesangioproliferative GN were examined by immunofluorescent microscopy (IFM) without knowledge of the LM or the clinical findings at the time of investigation. Correlation of the fluorescent findings in the renal corpuscles to the clinical symptoms, and to part the clinical course, showed: 1 a significantly lower frequency of complete remission in patients with deposits of IgG-IgA/C₃ and a higher frequency of complete remission in patients with deposits of IgG-IgA/C₃ 2 that IgG-IgA/C₃ was significantly more common in patients with deposits of IgG-IgA/C₃ than other deposits, and IgA/C₃ was more commonly localized solely to the mesangium than other deposits, and IgA/C₃ was more commonly localized along the basement membrane of the capillary loops than other deposits. 3 that patients with elevated blood pressure and at the same time reduced kidney function all had deposits of immunoglobulin and C localized to both the mesangium and the capillary loops in the glomeruli. 4 that those patients treated with corticosteroids and/or cytotoxic drugs had a greater tendency to complete remission. 5 that the effect of immunosuppressive treatment was independent of whether glomerular deposits of immunoglobulin and C₃ were present or not. Apart from that no significant association was found between immunoglobulin/C₃ deposited in glomeruli and the clinical symptoms of GN or the clinical course. In patients presenting with symptoms of GN less than 3 months before biopsy, deposits of properdin were found to be four times more common than in patients where disease was present for more than 3 months before biopsy. Immunoglobulin and C₃ were demonstrated together in only 72% of the biopsies, and in 15% neither deposits of immunoglobulin nor C₃ were found. The demonstrated deposits of different immunoglobulin combinations in glomeruli could be used as the basis of an immunological classification in patients with mesangioproliferative GN on LM. Any such classification, however, only seems to be on yet another descriptive level with the same correlative problems as the LM diagnosis because of the lacking relationship of the IFM findings to symptoms, clinical course and results of therapy.

Key words: Immunofluorescent microscopy, kidney disease, glomerulonephritis.

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Received 21st 78 Accepted 8 78

Today IFM is used increasingly as an aid in examination of kidney biopsies in human kidney disease. The immunological findings in GN are to the degree analogous with those demonstrated in immunological experimental models. In these the

CN is caused by either well-defined circulating antigen-antibody complex deposition in glomeruli or by circulating antibodies directed against the glomerular basement membrane, (McCuskey *et al.* 1960, Dixon 1968).

In contrast to the experimental models the

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range 2½ months (1-3 months) in 1/3 of the young, and 22½ months (3 months-17 years) in 1/3 of the patients.

Prior to biopsy 18% of the patients were on treatment, and 5 (4%) patients were using cytotoxic drugs.

One week after biopsy 34% were on corticosteroid and/or cytotoxic treatment.

3 patients died in the observation period (2 with bacterial infection, 1 with intractable uraemia).

3 patients with progressive uraemia began

TABLE 1 Terminology and Definitions

Terminology	Abbreviation	Definition
Fluorescence type () (+)		Bright fluorescence weak, moderate and heavy
Absent	-	Complete absence of fluorescence
Capillary basement membrane	(Mbm)	Glomerular intercapillary region
Capillary basement membrane	(Cap)	Within/along the capillary basement membrane
Bowman capsule	(Bm)	In Bowman's capsule
Distribution Generalised	(G)	All glomeruli in specimen
Focal	(F)	One/some glomeruli in specimen
Global	(Glo)	Including all portions of the individual glomerulus
Segmental	(S)	Including only parts of the individual glomerulus
Pattern Granular	(Gr)	Fine/coarse granular fluorescence
Linear	(L)	Linear continuous or discontinuous fluorescence within/along the base- ment membrane

TABLE 2 Deposits Correlated to the Localisation in Glomeruli

Deposits	No Pts	No	No	Cap
				Cap
		%		
Ig[G.M.A.] + C ₃	15	0	80	20
Ig[G.A.] + C ₃	4	24	63	13
Ig[A] C ₃	1	67	33	0
Ig[M.A.] + C ₃	3	0	100	0
Ig[G.M.] + C ₃	14	0	93	7
Ig[M] C ₃	6	11	50	50
Ig[G] + C ₃	11	18	55	27
C ₃	8	0	75	5
Ig[G][M][A][G.A.]	6	17	83	0
Total	90	1	71	17

P<0.001

P<0.01

longterm haemodialysis. 4 patients received intercurrent dialysis followed by remission, though with a reduced yet stable kidney function.

Elevated BP was seen in 27 patients (25%) only 2 were under 14 years of age.

A clinical history of upper respiratory tract infection or otitis media was present in 32 patients (30%).

In 23 of these the ASO > 600 and in all other patients in the study the ASO was normal or only slightly elevated. In 21 patients (20%) GN was secondary to other primary disorders (11 Henoch-Schönlein purpura, 5 SLE, 4 possible cases of systemic disease, 1 pernicious podagra).

Fluorescent Microscopy Findings

Definitions and the terminology used are shown in Table 1.

In 16 biopsies no deposits were demonstrated.

In 90 biopsies with deposits, the distribution was generalised except in 2 cases, where it was focal. The average number of glomeruli investigated was 5 (Range 2-18).

The localisation of the demonstrated deposits appears in Table 2.

IgG-IgA/C₃ differed significantly from other deposits, being more often localised solely to the mesangium. IgM/C₃ was found more often than other deposits localised solely to the basement membranes of the capillary loops on the endothelial side.

No correlation was found between other deposits and their localisation.

Appearance of deposits. In all glomeruli with positive fluorescence findings granular deposits were

antigen in human GN has only been demonstrated in a few instances and in these it has been best demonstrated as DNA in GN secondary to systemic lupus erythematosus (SLE) by *Koffler et al* (1967). The lack of demonstration of the antigen in these presumed immune complexes in human GN does not, however, exclude that analysis of the different deposits in immunoglobulin combinations, as well as assessment of their pattern and localisation can lead to a more exact characterisation of this glomerulopathy which has identical LM changes.

In the present study the IFM findings in 114 biopsies, classified as generalised mesangioproliferative GN, are correlated to the clinical data.

The investigations were performed in the Department of Clinical Chemistry, Kommunehospitalet, Copenhagen from 1972-1976.

MATERIAL AND METHODS

Selection of Patients

To be included in the study patients had to have been under observation for at least 3 months, and to have had at least one of the following symptoms in an afebrile period shortly before kidney biopsy:

Haematuria, macroscopic or microscopic, with at least 2 erythrocytes in the urine/high power field (*Larcom & Carter* 1948), without any urological disease.

Proteinuria, more than 0.5 g/day (*Reiman & Levinsky* 1971).

On LM the biopsies had to have been classified as generalised mesangioproliferative GN characterised by mesangial and endothelial cell hypercellularity. Examination of the biopsies had to be based on at least 10 glomeruli, whilst for IFM at least 7 were required. 114 biopsies fulfilled the above conditions, 8 were re-biopsies.

Elevated blood pressure (BP) was defined as diastolic ≥ 100 mm Hg and the term nephrotic syndrome was used when the serum albumin ≤ 20 g/l (*Scremer* 1971) with proteinuria > 5 g/day (*Robson* 1967).

Infection antibiotic treated upper respiratory tract infections up to 6 weeks before kidney disease presented and/or antistreptolysin titre (ASO) > 200 .

Reduced kidney function serum creatinine > 1.5 mg% or creatinine or ^{51}Cr EDTA clearance < 1 ml/sec.

Method

The needle biopsy was immediately divided under a stereomicroscope for study by LM and IFM.

The IFM investigations were carried out without knowledge of clinical data or the result of the LM examination. Tissue for IFM was frozen using dry ice, embedded in Tissue TekR-gelatin (Ames Laboratory) and sections of $1 \mu\text{m}$ were cut at -74°C on a Leitz histocryotomeR with an automatic section remover.

Throughout the investigation all kidney sections were examined by a direct immunofluorescent staining technique using FITC-conjugated rabbit or goat antisera

specifically reactive to human IgG, IgM, IgA as well as complement C_3 and fibrin/fibrinogen.

From January 1974 to December 1975 these investigations were supplemented with anti-IgD, IgE, C_4 , C_{1q} and anti-properdin. Details of the procedure and controls have been described previously (*Larsen* 1978).

A Zeiss universal fluorescent microscope with transmitted light and Dark Field Ultracondenser N.A. 1.2/1.4 and objective 0.75 to 1.0 N.A. $\times 40$ and eye piece $\times 10$ kpl was used in the study with HbO 700 W/4 pressure mercury lamp and filter (Rigard & Olsen 1971).

Light microscopy examination (Dr Claus Bruun) see *Larsen* (1978).

Statistical Methods

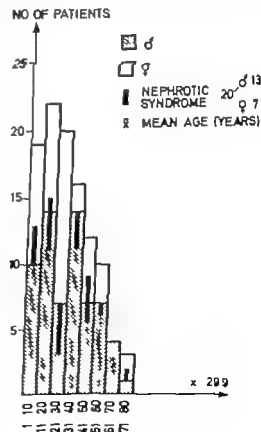
The correlation between the IFM findings and the clinical data was tested by Chi square test.

RESULTS

Age and sex distribution, as well as the occurrence of nephrotic syndrome in the different age groups, is shown in Fig 1. The length of time between presentation of kidney disease and biopsy was on

FIG 1

106 PTS. GENERALIZED MESANGIO PROLIFERATIVE GLOMERULONEPHRITIS
AGE, SEX AND NEPHROTIC SYNDROME



age 2½ months (½–3 months) in 1/3 of the cases, and 22½ months (3 months–17 years) in 1/3 of the patients.

Prior to biopsy 18% of the patients were on corticosteroid treatment, and 5 (4%) patients were receiving cytostatic drugs.

One week after biopsy 34% were on corticosteroid and/or cytostatic treatment.

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3 patients with progressive uraemia began

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Fluorescence		
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Granular	(Gr)	Fine/coarse granular fluorescence
Linear	(L)	Linear continuous or discontinuous fluorescence within/along the basement membrane

TABLE 2 *Deposits Correlated to the Localisation in Glomeruli*

Deposits	No. Pts	Mes	Mes + Cap	Cap
		%		
Ig[G.M.A.] + C ₃	15	0	80	20
Ig[G.A.] + C ₃	24	24	63	13
Ig[A.] + C ₃	3	67	33	0
Ig[N.A.] + C ₃	3	0	100	0
Ig[G.M.] C ₃	14	0	93	7
Ig[M.] + C ₃	6	0	50	50
Ig[G.] + C ₃	11	18	55	27
C ₃	8	0	75	25
Ig[G][M][A][G.A.]	6	17	83	0
Total	90	12	71	17

P < 0.001

P < 0.01

long-term haemodialysis, 4 patients received intermitent dialysis followed by remission, though with a reduced yet stable kidney function.

Elevated BP was seen in 27 patients (25%), only 2 were under 14 years of age.

A clinical history of upper respiratory tract infection or otitis media was present in 32 patients (30%).

Of 23 of these the ASO > 600 and in all other patients in the study the ASO was normal or only slightly elevated. In 21 patients (20%) GN was secondary to other primary disorders (11 Henoch-Schönlein purpura, 5 SLE, 4 possible cases of systemic disease, 1 periarthritis nodosa).

Fluorescent Microscopy Findings

Definitions and the terminology used are shown in Table 1.

In 16 biopsies no deposits were demonstrated.

In 90 biopsies with deposits, the distribution was generalised except in 2 cases, where it was focal. The average number of glomeruli investigated was 5 (Range 2–18).

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No correlation was found between other deposits and their localisation.

Appearance of deposits in all glomeruli with positive fluorescent findings granular deposits were

TABLE 3 106 Patients with Proliferative Glomerulonephritis. Deposits Correlated to Clinical Information

Deposits	No. Pat.	Haematuria	Proteinuria	Nephrotic Syndrome	BP elevated	GFR decreased	Infection	Systemic Disease	Compl remission	Duration of disease before Biopsy (Months)		X, Months	Fibrin/toned	Deposits
										≤ 3	> 3			
%														
Ig [G,M,A] + C ₃	15	60	93	7	20	20	20	33	40	27	73	18	8/15	
Ig [G,A] + C ₃	24	83	88	13	21	8	33	17	8	33	67	12.5	6/24	
Ig [A] + C ₃	3	100	66	0	33	0	100	0	0	66	33	5.7	2/3	
Ig [M,A] + C ₃	3	100	100	33	0	33	0	0	33	33	67	18.3	7/3	
Ig [G M] + C ₃	14	64	100	36	43	22	29	29	50	50	50	10.9	3/14	
Ig [M] + C ₃	6	66	100	17	50	0	20	33	0	0	100	13.5	7/6	
Ig [G] + C ₃	11	63	91	36	27	9	27	18	18	45	55	8.6	4/11	
C ₃	8	75	87	0	13	13	25	25	25	0	100	16.3	7/8	
Ig [G][M][A][G,A]	6	100	100	17	33	33	50	17	0	33	67	9.0	0/6	
No deposits	16	50	75	25	19	13	31	6	31	44	56	12.3	5/16	
Total	106	71	90	19	25	14	30	20	74	34	66	12.5	34/106	9

= p < 0.01

= p < 0.001

 \bar{x} = Mean obs. periode.

found which were classified as finely or coarsely granular. Those classified as finely granular could be very often related to properdin or C_{1q}, for these in all instances were noted to be finely granular. In addition granular size was found to be dependent on section thickness: deposits in 1 μ m and 4 μ m sections from the same biopsy appeared more finely granular in the thinner sections.

Continuous linear deposits could not be demonstrated, but in 27% of biopsies interrupted linear deposits were seen along with granular deposits.

(11% immunoglobulin with C₃, 9% immunoglobulin without C₃, 7% C₃ alone).

The IFM findings of IgD, IgE, C_{1q} and C₄ are not shown in the tables but are mentioned below.

IgD 73 biopsies were tested but IgD was demonstrated in only one patient where it was deposited together with IgA/C₃. (Biopsy 2 weeks after presentation of GN: no systemic disease).

IgE 42 biopsies were tested: none was positive.

C_{1q} 27 biopsies were tested and C_{1q} was demonstrated in 6 of them. In one case C_{1q} was

TABLE 4 Localisation of Deposits Correlated to Clinical Information

Localisation of Deposits	No Pat.	Haematuria	Proteinuria	Nephrotic Syndrome	BP elevated	GFR decreased	Infection	Systemic Disease	Compl remission
						%			
Mes.	11	91	100	18	18	0	45	27	9
Mes. + Cap	64	87	90	19	33	20	30	22	26
Cap.	15	73	93	13	6	0	70	20	13
Total (%)	90	77 (85)	83 (92)	16 (18)	24 (27)	13 (14)	27 (30)	20 (22)	70 (22)

p < 0.01

p < 0.001

TABLE 5 *Eight Biopsies and Re-biopsies: Deposits and Localisation in Complete Remission*

Case Code	Interval between biopsies (weeks)	Deposits				Localisation			Complete remission	Progression
		G	M	A	C ₃	Mes + Cap	Cap	Mes		
1 44	6	+	+	+	+	+				
33B		-	-	-	-				+	-
2 95	6	-	-	-	+	+			-	-
95B		+	+	+	+	+				
3 105	6	+	+	+	+	+			-	+
105B		+	+	+	+	+				
4 185	6	+	+	+	+	+			-	-
185B		+	-	+	+	+				
5 186	24	+	-	+	+			+	-	-
186B		+	-	+	+	+				
6 231	11	+	-	+	+	+			-	-
231B		-	-	-	-		+			
7 370	11	+	+	-	+	+			-	+
370B		-	-	-	-					
8 452	15	-	-	-	-				-	-
452B		+	-	-	+	+				

2.M.A. Immunoglobulin Classes C₃ = Complement C₃ B = Re-biopsy

toed together with C₄ properdin, and IgG/IgA, in 1 case together with other immunoglobulin combinations and C₄ but without IgA.

C₄ 44 biopsies were tested and C₄ was demonstrated in 10 cases, in 6 biopsies it was co-localised together with C₃, whilst in 4 biopsies it was shown to be the only glomerular deposit.

Properdin 31 biopsies were tested and properdin was demonstrated in 9 of these. Table 3 shows with black immunoglobulin deposits properdin was local.

Fibrin/fibrinogen all biopsies were tested and 34 (30%) demonstrated deposits. The deposits were solely granular or linear and localised simultane-

ously both to the mesangium and capillary loops in 28 cases, in 6 biopsies the deposits were localised solely to the mesangium.

The distribution of fibrin deposits appears in Table 3. No significant correlation was found.

In 31 biopsies with antibodies, fibrin deposits were found in 12 testates (39%).

Bowman's capsule in 12 biopsies focal deposits of immunoglobulin and C₃ were found. Immunoglobulin was deposited in an interrupted linear pattern in the basement membrane. Deposits of complement were demonstrated in 8 cases, all were granular and found in 4 biopsies together with immunoglobulin, whilst in 4 other cases this was

TABLE 6 *7 of 8 Biopsies: Correlation of Deposits to Concentration of Serum-Immunoglobulins and Serum-C₃ at Time of Biopsy*

Serum-Ig glob conc g/l		Deposits in glomeruli C ₃										
Ig	Standard deviation	M	GM	GM	GA	GA	GA	GA	A	GA	G	No Deposits
IgG	4-12	5.5	16.0	2.0	9.5	10.0	10.5	11.5	6.9	11.8	11.0	1.5 (0.5)
IgM	0.4-1	1.2	2.9	1.3	0.6	0.4	1.8	1.6	1.3	2.4	0.9	0.8 (1.4)
IgA	0.5-3	3.4	1.9	1.7	2.6	2.9	2.9	3.0	1.6	4.8	1.8	1.4 (3.1)
C ₃ %	70-136%	105	97	143	125	100	94	123	100	116	135	118 98

the only deposit. No deposits were demonstrated in the epithelium of Bowman's capsule.

Fluorescent Microscopy Findings Correlated to the Clinical Data

Correlation between glomerular deposits of immunoglobulin and the clinical symptoms and course appear in Table 3. With deposits of IgG-IgA/C₃ there was significantly less tendency to complete remission ($p < 0.01$) and with IgG-IgM/C₃ complete remission was more frequent ($p < 0.001$). Apart from this there was no significant correlation between the IFM findings and the clinical symptoms and course of the disease.

The correlation between localisation of deposits and the clinical data appears in Table 4. The only significant finding was an increased incidence of reduced kidney function and elevated BP in patients with deposits localised simultaneously to both the mesangium and along the capillary loops.

The appearance of the deposits, whether finely granular coarsely granular or interrupted linear showed no correlation to the duration clinical symptoms or course of the disease.

Properdin deposits were demonstrated in 6 of 11 biopsies examined (55%), where the disease had been present for up to 3 months prior to biopsy. In patients where the disease had been present for more than 3 months before biopsy properdin deposits could only be demonstrated in 3 of 20 biopsies examined (15%).

The IFM findings in 8 re-biopsies appear in Table 5 where they are compared with the first biopsy and correlated to the clinical course. From this it appears that deposits cannot always be demonstrated at the time of biopsy in spite of clinical evidence of GN being present, and that deposition occurs later in the course of the disease. It is also of note that neither presence or lack of deposits gives any direct indication of the clinical course at the time of biopsy.

In 12 patients serum immunoglobulins and C₃ were measured at the time of biopsy and in Table 6 these are compared with glomerular deposits of immunoglobulin. C₃ was within normal limits in all patients.

The individual serum immunoglobulin fractions were not specifically increased or reduced in relation to the deposited immunoglobulins.

However the mean value of serum IgA was at the upper limit of normal in patients with glomerular deposits of IgA. This was not the case in patients with deposits of other immunoglobulin types. The study was too small to draw definite conclusions about these findings.

One week after biopsy 36 patients (34%) were on corticosteroid or cytostatic treatment. Complete remission occurred in 25 patients (24%) during the period of study. Correlation between the IFM findings and complete remission in both treated and untreated patients appears in Table 7.

Complete remission was seen in 36% of treated

TABLE 7 Patients with Proliferative Glomerulonephritis: Fluorescent Microscopic Findings in Glomeruli Correlated to Complete Remission in Treated and Untreated Patients

Deposits	No	Treated		Untreated	
		Compl	remission	Compl.	remission
Ig [G,M,A] + C ₃	15	6	2	9	4
Ig [G,A] + C ₃	24	9	1	15	1
Ig [A] + C ₃	3	0	0	3	0
Ig [M,A] + C ₃	3	1	1	2	0
Ig [G,M] + C ₃	14	8	5	6	2
Ig [M] + C ₃	6	2	0	4	0
Ig [G] + C ₃	11	2	1	9	1
C ₃	8	1	0	7	2
Ig [G][M][A][G,A]	6	0	0	6	0
No deposits	16	7	3	9	2
Total	106	36	13	70	12

patients compared with 17% of untreated patients. This was significantly higher in the treated group of patients ($p < 0.05$) ($X^2 = 3.75$).

Complete remission occurred just as frequently (34%) in the groups of treated patients with deposits of immunoglobulin/C₃ as in those without these deposits.

The same held true in patients who were not treated in the group of patients with deposits of immunoglobulin/C₃ and the group without these deposits; complete remission occurred in 17% and 18% respectively.

Treated patients, therefore, went into complete remission more often than those who were untreated, irrespective of whether glomerular deposits of immunoglobulin/C₃ were present or not.

DISCUSSION

The most common immune mechanism in human GN is deposition of circulating immune complexes (antigen-antibody complexes) in glomerular tissue (Dixon 1968), with an excess of antigen (Dixon 1972).

In generalised proliferative GN whether primary (Farkholder *et al.* 1969), or secondary to SLE (Depner *et al.* 1972), and Henoch-Schönlein purpura (Utzinger *et al.* 1968), granular deposits of immunoglobulins and C₃ can be demonstrated in glomeruli, and they are generally considered to be immune complexes responsible for GN. As the relatively uniform morphological glomerular lesions cannot be correlated to any specific clinical picture, it might be thought that the kidney lesion in these circumstances is caused by the same pathogenic mechanism. However the immunopathological findings presented here, together with those reported in the quoted literature, suggest a heterogeneous pathogenesis because of the variation in types of immunoglobulins deposited and their localisation. Any such appraisal of the pathogenic relationship is, however, burdened by one important drawback: the antigen, which must be presumed to be of essential importance in the pathogenesis, has only been demonstrated with certainty in a few instances in human GN (Kuselman & McIntosh 1971; Koffler *et al.* 1967). However a number of bacteria and viruses are presumed to act as antigens, inducing complex formation in human GN (McChesley 1974). In the present study the aetiology of GN was known in only 3 patients with rheumatoid arthritis, for symptoms of the kidney disorder began 2-4 weeks after gold therapy was started. Complete remission occurred within 3 months of stopping treatment. All 3 patients had deposits of IgG-IgM/C₃ in the mesangium and along the endothelial side

of the capillary loops. Removal of the gold salt, with ensuing complete remission, may be the reason why the group of patients with deposits of IgG-IgM/C₃ have a significantly higher frequency of complete remission than patients with other deposits.

In 21% of the patients, GN was probably of streptococcal origin, in agreement with a study of 55 children with generalised proliferative GN (Hafab 1970). On the other hand systemic disease was twice as frequent in this present study (70% compared with 9%), which may be due to the older patients studied.

In 53% of the patients no cause could be demonstrated and the aetiology of the GN therefore remained unknown. Distribution of age and sex in this study and the percentage frequency of symptoms was in agreement with the clinical data reported by Bohle *et al.* (1976) in a review of 584 patients with proliferative GN.

The results of the IFM findings in the present study must be considered surprising.

No significant relationship was found between deposits of the different types of immunoglobulin and the clinical symptoms. All the symptoms which occurred in patients with deposits of immunoglobulin and C₃ also appeared in patients where these deposits could not be demonstrated.

The variation in deposition of the different classes of immunoglobulin points towards a heterogeneous pathogenesis, where the immunoglobulin portion of the complex, however, does not seem to have any dominating influence on the nature of the symptoms.

It is not possible to exclude that some of the demonstrated deposits may be purely secondary and therefore not an expression of a primary immunopathological process, for in 2 cases deposits of immunoglobulin and C₃ could not be demonstrated in the first biopsy in spite of obvious morphological changes and clinical symptoms of GN; yet these deposits were demonstrable in a later re-biopsy. Similar findings have been reported by Olsen *et al.* (1974). Furthermore, deposited immunoglobulin and C₃ is not always the mark of an immunological reaction for these can be demonstrated in kidney disease of non-immunological origin (Berger *et al.* 1971).

Cameron (1972) has raised the question of whether the presence of immunoglobulins shows anything more than involvement of immune mechanisms at a purely secondary or attendant level. At the same time, however, the possible importance of the secondary response as a contributing factor in the development of chronic disease cannot be discounted, (Urethane & Dixon 1965).

The appearance of the deposits, whether granular

or more coarsely granular and confluent, referred to as «lumpy», has been reported to be an expression of the degree of severity of GN (Koffler *et al* 1969). This could not be demonstrated here, but on the other hand the granular size was found to be dependent partly on section thickness and partly on what type of immunoglobulin was deposited. Interrupted linear deposits in the basement membrane of the capillary loops could not be related to duration of disease, symptoms or combination of symptoms. In contrast to Michael *et al* (1966) and Fish *et al* (1970), who only found linear deposits in either re-biopsies or in biopsies taken in a late phase of the disease.

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Of 73 biopsies tested for IgD this was demonstrated in only one case. The biopsy was taken less than 2 weeks after the disease started. The role of IgD is not clear but it has been demonstrated in proliferative GN together with cryoglobulin, (Tarantino *et al* 1973), and can be an indication of longterm antigen stimulation, (Tarantino *et al* 1974). Others have demonstrated IgD as linear deposits in linear nephritis together with IgG but only reactive IgG

could be demonstrated in the patients serum, (Kar *et al* 1975). All patients with reduced kidney function, and at the same time elevated blood pressure, had immunoglobulin deposits and C₃ localised both to the mesangium and the capillary loops. These patients were on corticosteroid and/or cytostatic treatment, and all patients had been ill for more than 5 months before a biopsy was taken. There can be several reasons for the specific localisation of deposits in these patients. Experimental studies have shown that both elevated BP and reduced filtration can be accompanied by changes in the glomeruli, which may be the cause of the irregular distribution of immune complexes, (Gerrnuth *et al* 1967). Steroid treatment can produce alterations in the localisation of deposits with migration from the glomerular basement membrane to the mesangium (Gerrnuth *et al* 1968). However it is not clear whether corticosteroid treatment produces functional changes in the capillary loops or in the amount or structure of immunoglobulin produced. Other patients without treatment showed the same distribution of deposits without demonstrable elevation of BP or reduced kidney function.

Complete remission was significantly more frequent in patients treated with corticosteroids and/or cytostatic drugs. Treatment was not randomised, and only patients with the most severe symptoms were treated, which therefore suggests that at least some confidence may be placed on the efficacy of treatment. Complete remission in treated patients was independent of whether glomerular deposits were present or not. Therefore the type of immunoglobulin deposited cannot be used as an indicator for or against using immunosuppressive therapy.

The effect of treatment was just as good in patients with and without glomerular deposits, and so a negative immunopathological result is no contraindication for immunosuppressive treatment. It is therefore pertinent to question just at which immune mechanism treatment is really aimed, for cell mediated immunity can also be seen in proliferative GN (Mahieu *et al* 1972).

From the results of this study it may be concluded that glomerular deposits of different combinations of immunoglobulins can be used as a basis for an immunopathological classification in patients with proliferative GN on LM. However such a classification only seems to be on yet another descriptive level with the same correlative problems as the LM diagnosis, because of the lacking correlation of the IFM findings to symptoms, clinical course and results of therapy.

I gratefully acknowledge the financial support of The Danish Medical Research Council (J. nr. 312 2669) and King Christian X Foundation. My thanks are due to Hanne Pedersen, Karin Søberg and Birgit Hoff for skilful technical assistance.

I am grateful to Dr. Claus Bruus for use of the results of light microscopy of the kidney biopsies.

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